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BACHRACH U. (1957). *J. gen. Microbiol.* 17, 1–11

The Aerobic Breakdown of Uric Acid by Certain Pseudomonads

BY U. BACHRACH*

Department of Microbiology, The University of Reading

SUMMARY: Four pseudomonad isolates which could use uric acid aerobically as a sole C, N and energy source were isolated from poultry house deep-litter, droppings, and nearby soil. Organisms harvested from media containing uric acid can degrade uric acid completely, with the formation of CO_2 and NH_3. Allantoin, allantoic, glyoxylic and formic acids were completely oxidized.

The existence of micro-organisms able to decompose uric acid aerobically has long been known (see Stapp, 1920), but relatively little work has been reported on the biochemical pathways of this decomposition. The excrement of chickens contains a relatively large amount of uric acid (*c.* 5% of the dry weight, depending somewhat on the diet; information kindly supplied by Dr. J. Tinsley). In the deep-litter method of poultry husbandry the uric acid of the droppings rapidly disappears from the litter and there is often a marked evolution of ammonia. It seemed likely therefore that established deep-litter beds in poultry houses might well be a habitat from which micro-organisms capable of decomposing uric acid could easily be obtained; this proved to be so. It was decided to seek aerobic organisms which could use uric acid aerobically as sole source of carbon, nitrogen and energy in order to obtain micro-organisms with a rich array of enzymes capable of affecting uric acid. Elective cultures yielded bacteria which grew aerobically on a uric acid + mineral salts medium.

The decomposition of uric acid by these organisms was then explored by examining their O_2 uptake with uric acid and various related compounds which have been implicated in biological degradation of uric acid, as substrates. These compounds are shown in Fig. 1, which indicates a pathway of uric acid decomposition compatible with the evidence so far obtained with the pseudomonads used in the present work. The existence of enzymes which catalyse some of the chemical steps in the pathway shown in Fig. 1 is well known (see Laskowski, 1951), one or more of these enzymes having been observed in many different forms of life. The pseudomonads isolated in the present work are able to carry the aerobic degradation of uric acid the whole way to carbon dioxide and ammonia. This paper reports a preliminary examination of their metabolism of uric acid.

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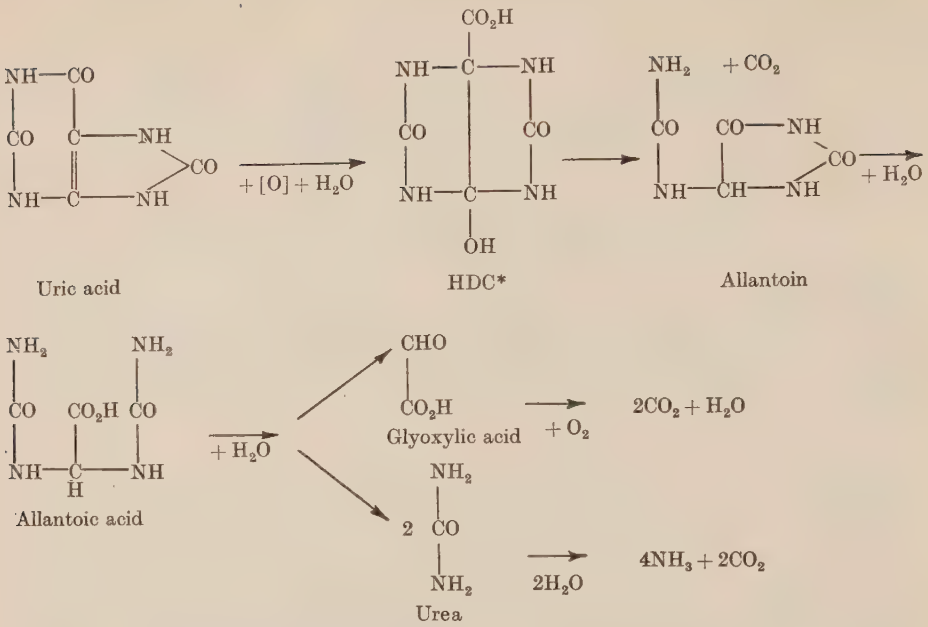

Fig. 1. Probable pathway of aerobic breakdown of uric acid by the pseudomonads used. Overall reaction: $C_5H_4N_4O_3+3[O]+4H_2O \rightarrow 5CO_2+4NH_3$. * HDC = compound made by the method of Schuler & Reindel (1932); for systematic name see Dalgleish & Neuberger (1954).

Isolation and characterization of organisms

The elective medium. This was made as follows: a solution of 5 g. KH_2PO_4/100 ml. tap water, adjusted to pH 7·3–7·5 by adding NaOH solution was filtered and autoclaved at 15 lb./sq.in. for 20 min. Sterile uric acid powder (sterilized by heating at 160° for 1 hr.) was then added aseptically to the phosphate solution to a final concentration of *c.* 0·5 g./100 ml., the liquid being shaken to disperse the powder. This suspension was then dispensed aseptically in 10 ml. vol. into sterile 6 in. × 1 in. rimless tubes closed by loosely fitting aluminium caps (Oxoid).

Samples were taken at the poultry research unit maintained at its Lane End Farm by the Department of Agriculture (University of Reading) as follows: deep-litter (from below the surface); droppings on the deep-litter; soil from the field surrounding the poultry houses. Portions of these samples were added to tubes of the elective medium which were sloped to give maximum exposure to air and incubated at 25°.

After four serial subcultures in elective medium, cultures were plated on solid medium of the basal composition described by Stapp (1920); this contained agar, uric acid, potassium and sodium phosphate, calcium chloride, magnesium sulphate, sodium chloride, ferric chloride, made up in distilled water. Colonies were picked and inoculated to the uric acid + phosphates liquid medium in tubes. Subcultures were made in the same medium to establish that the organisms so obtained could grow aerobically in serial subculture in a medium which contained effectively only uric acid as source of C, N and energy. Eventually four isolates were so obtained (one from soil, 2 from deep litter, 1 from droppings) and were examined for their bacteriological characters.

Characters of the isolates. The four isolates (A, B, C, D) showed the following characters: Gram-negative; motile; polar flagella; rods (except D which was cocco-bacillary); indole-negative; Voges-Proskauer negative; gelatin not hydrolysed (5 days); litmus milk alkaline (7 days) no clotting; non-lipolytic (margarine, tributyrin). Sugars in general gave no acid or gas, though isolates B and C gave acid with glucose. Nitrate was reduced to nitrite by isolates A and C. Isolates B and C gave a greenish fluorescent water-soluble pigment; A and D did not. Growth was very poor or absent at 37° with isolates A, B and C, while isolate D grew fairly well; there was no growth at 40°. They were classified as species of *Pseudomonas*, and not further characterized; they are clearly closely related. In the metabolic work not all four isolates were always used, but in replicate experiments with different isolates usually no essential differences were observed.

Materials and methods used in the metabolism experiments

For the metabolic studies organisms were usually grown on a complex medium containing uric acid, in order to obtain larger yields of organisms than those given by growth in the minimal uric acid + mineral salts medium. No marked differences were observed between the organisms grown on the two kinds of media. The complex medium consisted of: peptone (Evans), 10 g.; yeast extract (Oxoid), 3 g.; NaCl. 7·5 g.; agar (Kobe), 20 g.; 1000 ml. tap water; adjusted finally to pH 7·4. Sterile powdered uric acid to final concentration 0·2 % (w/v) was mixed into the molten agar to give a uniform suspension. This medium when solidified in the Petri dishes was opalescent, and the disappearance of uric acid during growth of the pseudomonads could be noted by the appearance of clear zones surrounding colonies. After 24 hr. of incubation at 25° the uric acid had disappeared and the organisms were collected, washed three times with 0·85 % (w/v) NaCl solution and resuspended at a suitable concentration in the same solution. These suspensions were stored when necessary at −20°, for not longer than 1 week. The concentrations of the suspensions used are noted in the Tables.

Substrates. Uric acid was used as the lithium salt (Davidson, 1938); allantoin as the potassium salt (Young & Conway, 1942); allantoic acid was prepared from allantoin according to Hermanowicz (1948). The compound intermediate between uric acid and allantoin (see Fig. 1) is called 'hydroxy-acetylene-diureido-carboxylic acid' in the earlier literature; its systematic name is given by Dalgleish & Neuberger (1954). It will be referred to here as HDC; the sample used was prepared from uric acid according to Schuler & Reindel (1932). Glyoxylic acid was supplied by Light and Co. (Poyle Estate, Colnbrook, Bucks); the purines were from Roche Products Ltd. (Welwyn Garden City).

Analytical procedures. Oxygen uptake and CO_2 output were measured by conventional Warburg manometric techniques with air as gas phase and bath temperature 30° (Umbreit, Burris & Stauffer, 1949); the 'direct' method of Warburg was used for CO_2 and correction was made for bound CO_2 by tipping acid at the end of the experiment. All manometric data quoted have been corrected for the endogenous rates.

For estimating various products the following analytical methods were used: uric acid by the method of Praetorius (1949) with a Unicam UV spectrophotometer (SP 500); purines spectrophotometrically (Hotchkiss, 1948); allantoin by the colorimetric method as modified by Franke, Taha & Krieg (1952); allantoic acid by the method of Di Carlo, Schultz & Kent (1953); glyoxylic acid as for allantoin but with the omission of the first step; ammonia by titration (Ma & Zuazaga, 1942) after steam distillation in a Markham still (Markham, 1942); urea by estimation of ammonia after treatment with urease; volatile fatty acids by steam distillation (Markham still) followed by titration of the distillate with 0·01 N-NaOH (phenolphthalein as indicator). Glyoxylic acid was identified by paper chromatography after its conversion to the corresponding 2:4-dinitrophenylhydrazone (Block, Durrum & Zweig, 1955); the solvent was butanol:ethanol:0·5 N-NH_4OH (70:10:20 by vol.). The R_f value for glyoxylic acid was 0·38.

Results of metabolism experiments

Oxidation of uric acid and related compounds by whole organisms

Adaptive enzymes. Preliminary experiments showed that the ability of the pseudomonads to decompose uric acid was not a constitutive property. Organisms grown on peptone water or on yeast-extract agar without added uric acid, oxidized uric acid only after a lag period (e.g. 120 min.). When these media were supplemented with uric acid (0·2 % w/v) the organisms so obtained oxidised uric acid without lag. The oxidation of uric acid by washed organisms was optimal at *c.* pH 7·4. All subsequent experiments were carried out in the presence of M/15 tris buffer at pH 7·3–7·5; similar results were obtained when phosphate buffer was used.

Uric acid as substrate. The degree of oxidation of uric acid and of some possible metabolic intermediates by uric acid-grown organisms is illustrated in Table 1. In every case about 3 atoms of oxygen were taken up for every mole of uric acid which disappeared. Estimation of urea at the end of experiment showed that about 2·3 μmole were left from 2·5 μmole of uric acid decomposed, thus, together with 9·9 μmole of CO_2 formed, accounting for all the carbon atoms present in the uric acid molecule (Table 2). In a larger scale experiment with 20 μmole uric acid and an amount of pseudomonad A equivalent to *c.* 60 mg. dry wt., the ammonia and urea formed during 6 hr were determined. The absorption spectrum showed that all the uric acid had disappeared; half the nitrogen of the uric acid appeared as ammonia and the other 2 atoms of nitrogen were accounted for as urea (Table 2). No volatile fatty acids were formed during this experiment.

Table 1. *Oxygen uptake by the uric acid-grown pseudomonads studied in presence of various substrates. Summary of results*

Each Warburg vessel contained 0·5 ml. substrate solution; 1·0 ml. of suspension of organisms (equivalent to 3–5 mg. dry wt.); 1·0 ml. of tris buffer pH 7·3; 0·2 ml. of 15% (w/v) KOH in centre well; total volume 3·2 ml. The gas phase was air. The values have been corrected for endogenous activity.

	Strain of pseudomonad			
	A	B	C	D
	O_2 uptake (μmole)			
Substrate (μmole)				
Uric acid, 5	7·4	7·0	7·35	6·9
Allantoin, 5	4·2	—	—	—
Glyoxylic acid, 5	4·6	5·3	4·75	5·2
Formic acid, 10	4·75	5·1	4·85	5·15

Intermediate compound HDC. Schuler & Reindel (1932), when studying the oxidation of uric acid by alkaline permanganate, isolated the silver salt of a symmetrical compound which was probably the intermediate compound HDC (see Fig. 1.). Subsequently, Cavalieri & Brown (1948) showed, by labelling the nitrogens of the pyrimidine ring of uric acid, that a symmetrical intermediate was formed during the oxidation of uric acid with alkaline permanganate. Klemperer (1945) suggested that a symmetrical compound might be obtained by the action of liver uricase on uric acid, and Brown, Roll & Cavalieri (1947) showed, by feeding rats with labelled uric acid, that the *in vivo* oxidation of uric acid involved a symmetrical intermediate.

Table 2. *Products of oxidation of uric acid and glyoxylic acid by pseudomonad A grown in presence of uric acid*

Experimental conditions as in Table 1, but for CO_2 estimations 0·2 ml. of 2N-HCl was tipped from side arm, and distilled water was in centre well.

Substrate (μmole)	O_2 taken up (μmole)	CO_2 evolved (μmole)	Urea formed (μmole)	NH_3 formed (μmole)
Uric acid, 2·5	3·85	9·9	2·3	—
Uric acid, 20·0*	—	—	20·25	39·5
Glyoxylic acid, 10·0	9·2	18·1	0	0

* In this experiment a suspension of organisms equivalent to *c.* 60 mg. dry wt./ml. was used.

It was therefore thought worthwhile in the present work to see whether a micro-organism which decomposed uric acid would also metabolize HDC. Free HDC, which was obtained by the addition of sodium chloride to the silver salt of HDC, was added to peptone water and inoculated with pseudomonad A; two-thirds of the HDC disappeared after 3 days while an uninoculated control of HDC in peptone water remained unchanged. Free HDC was also tested as substrate for pseudomonad A in manometric experiment. An uptake of oxygen was observed, but since the exact amount of HDC added was not known, the ratio O_2 uptake/mole HDC used could not be calculated.

Allantoin and allantoic acid. Pseudomonad A which had been grown on yeast extract medium + allantoin (0·2 %, w/v) oxidized allantoin, allantoic acid and glyoxylic acid without lag, but uric acid and xanthine were oxidized only slowly (Fig. 2). This suggests that allantoic acid and glyoxylic acid are intermediates in the breakdown of allantoin by this organism, according to the concept of simultaneous adaptation (Stanier, 1947). When the organisms were grown on a medium containing uric acid they oxidized allantoin (Table 1) without lag; this oxidation required about 1 mole oxygen/mole substrate.

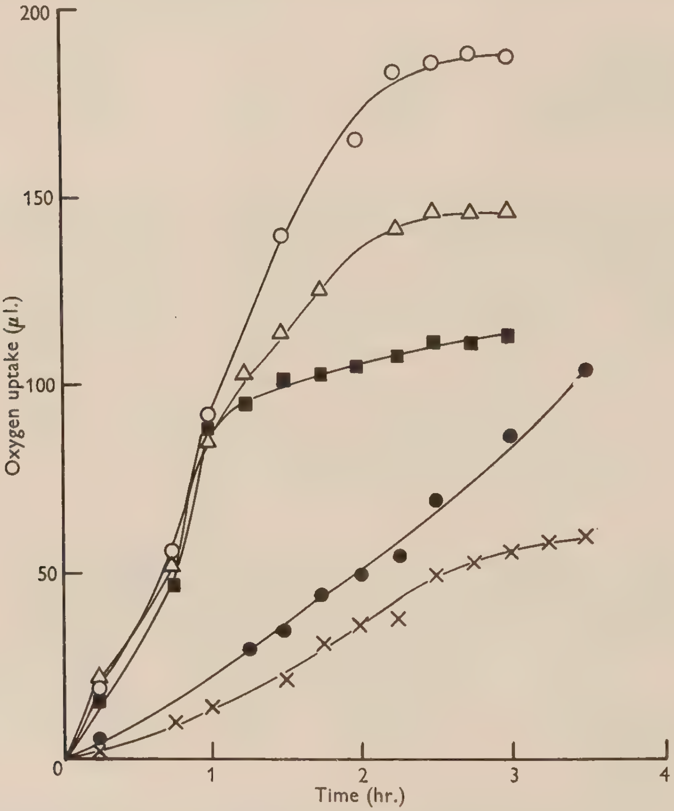

Fig. 2. Oxidation of various substrates by washed Pseudomonad A grown on allantoin. Each Warburg vessel contained the equivalent of 4 mg. (dry wt.) of organisms; 8 μmole glyoxylic acid, -○-○-; 6 μmole allantoic acid, -△-△-; 5 μmole allantoin, -■-■-; 5 μmole xanthine, -●-●-; 5 μmole uric acid, -×-×-. Other conditions as given in Table 1.

Glyoxylic acid and formic acid. Table 1 shows that during the oxidation of glyoxylic acid by the uric acid-grown organisms about 1 mole oxygen was taken up for every mole of glyoxylic acid which disappeared. During this oxidation 2 moles of carbon dioxide were evolved per mole glyoxylic acid which disappeared (Table 2) thus showing a complete oxidation.

When it was found that glyoxylic acid was completely oxidized by these pseudomonads the possible intermediates of its oxidation were considered.

It seemed possible that oxalic acid (Franke & Hahn, 1955) or formic acid (Campbell, 1954); Nakada & Weinhouse, 1953) might be an intermediate. Oxalic acid was not oxidized, but formic acid was oxidized completely by these pseudomonads (Table 1). In every case 1 atom oxygen was taken up for each mole of formic acid which disappeared; carbon dioxide was evolved during the reaction.

Anaerobic experiments

Allantoin was shaken with uric acid-grown organisms (pseudomonad B) in Warburg flasks with a nitrogen gas phase. After 120 min. 6 μmole allantoic acid and 1·2 μmole glyoxylic acid were formed from 10 μmole of allantoin which disappeared. When allantoic acid was used as substrate under the same conditions, *c.* 1 μmole glyoxylic acid was formed from 5 μmole allantoic acid which disappeared. The formation of glyoxylic acid was confirmed by paper chromatography. The fate of glyoxylic acid was also studied under anaerobic conditions. The concentration of glyoxylic acid markedly decreased; no change of gas volume occurred during this incubation. These results show that allantoin is metabolized by pseudomonad B to allantoic and glyoxylic acids. The glyoxylic acid formed may further be metabolized without O_2 consumption.

Effect of inhibitors

The effect of various enzyme inhibitors on the oxidation of uric acid and some intermediates was investigated with uric acid-grown organisms (Table 3). Sodium azide and cyanide both inhibited the oxidation of uric acid, allantoin and glyoxylic acid by washed pseudomonad B organisms grown on uric acid. Colorimetric estimations showed that most of the allantoin and glyoxylic acid disappeared in the presence of the inhibitors, although the oxygen uptake was markedly inhibited.

Compounds resembling uric acid and allantoin in their chemical structure were tried as inhibitors; benzimidazole was effective, but hydantoin and glyoxaline had no effect. Iodoacetate and *p*-chloromercuribenzoate inhibited the oxidation of uric acid and glyoxylic acid. These inhibitors had no effect on the oxidation of formic acid.

2:4-Dinitrophenylhydrazine and semicarbazide, which react with carbonyl groups, inhibited the oxidation of uric acid.

While using the Thunberg tube technique to study the decomposition of glyoxylic acid it was observed that almost no reaction took place in the presence of *c.* 10^{-4}M-methylene blue. Manometric experiments showed that the methylene blue inhibited oxygen uptake with glyoxylic acid as substrate, while that with formic acid was unaffected (Table 3).

It is known that arsenite inhibits oxidative decarboxylation processes. Because of the possibility that glyoxylic acid was metabolized by such a reaction, arsenite was tested as an inhibitor; oxygen uptake with glyoxylic acid or formic acid as substrate was not thereby affected.

According to Tolbert, Clagett & Burris (1949) oxalic acid inhibits the oxidation of glyoxylic acid. Various concentrations of oxalic acid (10^{-2} to

2×10^{-1} M) were used but no inhibition of oxygen uptake was observed with allantoin, glyoxylic, formic or uric acids as substrate.

Borate inhibits xanthine oxidase (Roush & Norris, 1950) and this enzyme also oxidizes aldehydes. Borate at 10^{-1} M inhibited oxygen uptake with glyoxylic acid by pseudomonad B, but had no effect with formic acid as substrate (Table 3).

Table 3. *Effect of various inhibitors on* O_2 *uptake of uric-acid-grown pseudomonads*

0·5 ml. inhibitor solution was used. Other conditions as in Table 1.

		Oxidation substrates			
		Uric acid	Allantoin	Glyoxylic acid	Formic acid
Inhibitor	Concentration of inhibitor (M)	Degree of inhibition of O_2 uptake (%)			
Azide	5×10^{-3}	20	40	40	50
Azide	5×10^{-2}	80	100	—	—
Cyanide	5×10^{-3}	30	40	—	—
Cyanide	5×10^{-2}	100	100	—	—
Benzimidazole	1×10^{-2}	50	—	—	—
Benzimidazole	1×10^{-1}	—	100	—	—
Hydantoin	2×10^{-1}	0	—	—	—
Glyoxaline	1×10^{-2}	0	—	—	—
Iodoacetate	5×10^{-3}	100	—	80	0
p-Chloromercuribenzoate	5×10^{-3}	100	—	100	0
2:4-Dinitrophenylhydrazine	2×10^{-2}	100	—	—	—
Semicarbazide	5×10^{-2}	100	—	—	—
Methylene blue	3×10^{-4}	—	—	60	0
Arsenite	5×10^{-3}	—	—	0	0
Oxalate	1×10^{-2} to 2×10^{-1}	0	0	0	0
Borate	1×10^{-1}	—	—	75	0

From the results summarized in Table 3, it may be concluded that one of the steps in the oxidation of uric acid is catalysed by an —SH enzyme which is inhibited by iodoacetate or *p*-chloromercuribenzoate (cf. Scheer & Scheer, 1943, who found that cysteine activated the uricase of liver). The degradation of glyoxylic acid does not seem to be an oxidative decarboxylation process, for it was not affected by arsenite.

Oxidation of other compounds by the pseudomonads

Purines. Uric acid-grown organisms (pseudomonad A) were tested for ability to oxidize various purines. According to spectrophotometric measurements xanthine disappeared completely, a partial disappearance of hypoxanthine and adenine took place, while guanine was scarcely affected. Manometric experiments gave similar results (Table 4).

Pyrimidines. Uric acid-grown organisms (pseudomonad A) showed no significant oxygen uptake with alloxan or uracil as substrates.

Imidazoles. Hydantoin, parabanic acid, glyoxaline and benzimidazole were tested as oxidation substrates for uric acid-grown organisms (pseudomonad A);

no significant oxygen uptake was observed with any of these compounds. The possibility of a hydrolytic degradation of these compounds was considered, but no increase in the amounts of urea or ammonia were observed after incubating the organisms with hydantoin or parabanic acid.

Table 4. *Oxidation of various substrates by uric acid-grown pseudomonads*

Experimental conditions as given in Table 1; 5 μmole substrate used. Duration of experiment: 60 min.

Substrate		O_2 uptake (μl.)
Purines	Xanthine	154
	Hypoxanthine	132
	Uric acid	114
	Adenine	56
	Guanine	17
Pyrimidines	Alloxan	2
	Uracil	5
Imidazoles	Hydantoin	11
	Parabanic acid	8
	Glyoxaline	4
	Benzimidazole	17
Other substrates	Glycine	110
	Citric acid	50
	Oxalic acid	12
	Glycollic acid	7
	Methyl alcohol	6
	Formaldehyde	4

Other substances. Formaldehyde, methanol and oxalic acid were not oxidized (uric acid-grown pseudomonads A and B), nor was glycollic acid (pseudomonad B). Glycine and citric acid were oxidized by washed uric acid-grown organisms (pseudomonads A and B).

DISCUSSION

Simultaneous adaptation experiments and the tests performed under anaerobic conditions, by which the degradation products of uric acid were demonstrated, support the view that uric acid is completely degraded by the pseudomonads from deep-litter through the pathway shown in Fig. 1. Campbell (1954) isolated a pseudomonad from San Francisco Bay mud which oxidized allantoin completely. This organism also oxidized uric acid to carbon dioxide and urea; glyoxylic acid was an intermediate in the oxidation, and by the use of carboxyl-labelled glyoxylic acid it was found that the carboxyl carbon was converted to formate (Campbell, 1955). Franke & Hahn (1955) showed that uric acid was oxidized to oxalic acid, via glyoxylic acid, by resting *Pseudomonas aeruginosa*.

With the pseudomonads tested (A and B) in the present work no accumulation of oxalic acid was found, nor were oxalic acid or glycollic acid oxidized. These observations, together with the uptake of 2 atoms of oxygen/mole glyoxylic acid which disappeared, would seem to eliminate the possibility of the pathway described by Franke & Hahn (1955), in the present pseudomonads.

Under anaerobic conditions, or in the presence of azide, glyoxylic acid disappeared without consumption of oxygen; therefore a direct oxidation of glyoxylic acid to formate and CO_2 seems excluded. The fact that arsenite, a known inhibitor of oxidative decarboxylation (Baldwin, 1952), had no effect on the oxidation of glyoxylic acid, is also evidence against the occurrence of this reaction. Indirect evidence against an oxidative decarboxylation is also given by the fact that the degradation of glyoxylic acid by the pseudomonads studied was not affected by the addition of oxalic acid (contrary to the effect of oxalic acid on the oxidative decarboxylation of glyoxylic acid by plant tissue; Tolbert *et al.* 1949). This conclusion agrees with the findings of Campbell (1954) and suggests that glyoxylic acid metabolism by the present pseudomonads may occur by a mechanism like the phosphoroclastic splitting of pyruvic acid (Chantrenne & Lipmann, 1950), namely: $R.CO.COOH \rightarrow R.CO-\text{enzyme}+HCOOH$; or by a condensation of glyoxylic acid with succinic acid to form isocitric acid (Saz, 1954) which might then be oxidized.

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REFERENCES

Baldwin, E. (1952). *Dynamic aspects of Biochemistry*, 2nd ed. Cambridge University Press.

Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of paper chromatography and paper electrophoresis.* New York: Academic Press.

Brown, G. B., Roll, P. M. & Cavalieri, L. F. (1947). The *in vivo* oxidation of uric acid. *J. biol. Chem.* **171**, 835.

Campbell, L. L. Jr. (1954). The mechanisms of allantoin degradation by a Pseudomonas. *J. Bact.* **68**, 598.

Campbell, L. L. Jr. (1955). Oxidative degradation of uric acid by cell extracts of a pseudomonas. *Biochim. biophys. Acta*, **18**, 160.

Cavalieri, L. F. & Brown, G. B. (1948). The mechanism of the oxidation of uric acid studied with isotopic nitrogen as a tracer. *J. Amer. chem. Soc.* **70**, 1242.

Chantrenne, H. & Lipmann, F. (1950). Coenzyme A dependence and acetyl donor function on the pyruvate-formate exchanges system. *J. biol. Chem.* **187**, 757.

Dalgleish, C. E. & Neuberger, A. (1954). *J. chem. Soc.* 3407.

Davidson, J. N. (1938). The purification of uricase. *Biochem. J.* **32**, 1386.

Di Carlo, F. J., Schultz, A. S. & Kent, A. M. (1953). The mechanism of allantoin catabolism by yeast. *Arch. Biochem.* **44**, 468.

Franke, W., Taha, E. E. M. & Krieg, L. (1952). Purinoxydierende Fermente aus Schimmelpilzen. *Arch. Mikrobiol.* **17**, 255.

Franke, W. & Hahn G. E. (1955). Untersuchungen zum bakteriellen Purinabbau *Hoppe-Seyl. Z.* **299**, 15.

Hermanowicz, W. (1948). Formation of allantonic acid from allantoin. *Roczniki Chem.* **22**, 159. (Taken from *Chem. Abstr.* (1950) **44**, 1418/h.)

Hotchkiss, R. D. (1948). The quantitative separation of purines and nucleosides by paper chromatography. *J. biol. Chem.* **175**, 315.

Klemperer, F. W. (1945). Enzymatic oxidation of uric acid. *J. biol. Chem.* **160**, 111.

Laskowski, M. (1951). In *The Enzymes*, **1** (2), 946; ed. by Sumner, J. B. & Myrback, K. New York: Academic Press Inc.

Ma, T. S. & Zuazaga, G. (1942). Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. *Industr. Engng Chem.* (*Anal.*), **14**, 280.

Markham, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

Nakada, H. J. & Weinhouse, E. S. (1953). Studies of glycine oxidation in rat tissues *Arch. Biochem.* **42**, 257.

Praetorius, E. (1949). An enzymic method for the determination of uric acid by ultraviolet spectrophotometry. *Scand. J. clin. Lab. Invest.* **1**, 222.

Saz, H. J. (1954). The enzymic formation of glyoxylate and succinate from tricarboxylic acids. *Biochem. J.* **58**, xx.

Roush, A. & Norris, E. R. (1950). The inhibition of xanthine oxidase by borates. *Arch. Biochem.* **29**, 244.

Scheer, B. T. & Scheer, M. A. R. (1943). The activation of uricase by cysteine. *J. biol. Chem.* **150**, 359.

Schuler, W. & Reindel, W. (1932). Die Urikolyse. *Hoppe-Seyl. Z.* **208**, 248.

Stanier, R. Y. (1947). Simultaneous adaptation: A new technique for the study of metabolic pathways. *J. Bact.* **54**, 339.

Stapp, C. (1920). Botanische Untersuchung einiger neuer Bakterien-species, welche mit reiner Harnsäure oder Hippursäure als alleinigem organischen Nährstoff auskommen. *Zbl. Bakt.* (2 *Abt.*), **51**, 1.

Tolbert, N. E., Clagett, C. O. & Burris, R. H. (1949). Products of the oxidation of glycollic acid and L-lactic acid by enzymes from tobacco leaves. *J. biol. Chem.* **181**, 905.

Umbreit, W. W., Burris, R. H., & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism.* Minneapolis, U.S.A.: Burgess Publ. Co.

Young, E. G. & Conway, C. F. (1942). On the estimation of allantoin by the Rimini-Schryver reaction. *J. biol. Chem.* **142**, 839.

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Steel, R. & Walker, T. K. (1957). *J. gen. Microbiol.* **17**, 12–18

Celluloseless Mutants of certain *Acetobacter* Species

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SUMMARY: The occurrence has been studied of mutants which did not produce extracellular cellulose in cultures of two strains of *Acetobacter acetigenum* (NCIB 8132, NCIB 5346) and one strain of *A. xylinum* var. *africanum* (NCIB 7029). With about 150 glucose yeast-extract (GYE) medium static cultures of organism NCIB 8132, prepared over a period of about one year, cellulose pellicles were produced in each case. In contrast, the loss of cellulose-forming ability and the appearance of diffuse general growth were noted with 6 out of 26 tests made with static cultures in an ethanol yeast-extract (EYE) medium, 3 out of 905 isolates selected from platings of GYE shaken cultures, and 1 out of 3 tests made with EYE shaken cultures. By a 'mass-plating' technique it was found that mutants which did not produce cellulose pellicles ('celluloseless mutants') were present in cellulose-producing cultures. The two other cultures (NCIB 5346, NCIB 7029) underwent mutation spontaneously in GYE static cultures on several occasions.

The present work was undertaken to obtain mutants of *Acetobacter acetigenum* and *A. xylinum* var. *africanum* which did not form extracellular cellulose as did the parent organisms; these mutants will be referred to as 'celluloseless'. Schramm & Hestrin (1954) reported the occurrence of celluloseless mutants of *A. xylinum*; the mutants appeared when the wild-type organism was grown by serial transfer in 'swirl' cultures on a medium containing glucose, yeast-extract, peptone and salts. Creedy, Jowett & Walker (1954) isolated a celluloseless mutant of *A. acetigenum* when the parent organism was cultivated in the presence of sodium arsenite (0·0005 M). Further, Wright & Walker (1955) found that isolates incapable of forming cellulose were present when *A. acetigenum* was grown under agitated and aerated conditions. The present paper deals primarily with the occurrence of celluloseless mutants when the wild-type of *A. acetigenum* was grown under various cultural conditions.

METHODS

Organisms. Two strains of *Acetobacter acetigenum* (NCIB 8132, NCIB 5346) and one of *A. xylinum* var. *africanum* (NCIB 7029) were obtained from the National Collection of Industrial Bacteria, (NCIB), Teddington, Middlesex. All cultures were replated and re-isolated twice and examined microscopically for purity before use.

Media. Glucose yeast-extract (GYE) medium (pH 7·0) contained 3 % (w/v) glucose and 0·5 % (w/v) Difco yeast extract; for solid media 2 % (w/v) of agar was added. Ethanol yeast-extract (EYE) medium contained 2 % (v/v) ethanol and 0·5 % yeast extract, the sterile ethanol being added aseptically after sterilization of the basal medium. Tests were also made with ethylene glycol or *n*-propanol (both at 2 %, v/v) substituted for ethanol.

Peptone medium consisted of a 5 % (w/v) aqueous solution of Evans's peptone. Malt wort medium (pH 5·5) was prepared by the method of Tosic & Walker (1946). In the early stages of this work the stock cultures of the parent organisms were kept on GYE medium. However, as indicated later, malt wort was found to be better for this purpose and accordingly was used thereafter. Cultures were incubated at 30° except when otherwise stated.

Static cultures were made either with 30 ml. of medium in 100 ml. conical flasks or with 8 ml. of medium in test tubes (18 × 155 mm.).

Shaken cultures. The transfer procedure of Schramm & Hestrin (1954) was followed. Duplicate conical flasks (1500 ml.) which contained 100 ml. of the desired medium were inoculated from a 48 hr. GYE test-tube culture by dipping the cellulose pellicle in the medium. The flasks were placed on a reciprocating shaker (55 cycles/min.; 6·5 cm. stroke) in a room controlled at 28 ± 1°. At 3-day intervals transfers were made with a sterile wire loop from each of the duplicates to two sterile flasks of fresh medium, and one flask of each pair was incubated statically while the other was replaced on the shaker. According to Schramm & Hestrin (1954) the appearance of turbidity (due to diffuse growth of organisms) in the static cultures indicates the presence of celluloseless mutants. When turbidity was observed, the shaken cultures were plated on GYE agar medium and isolated colonies were picked to GYE liquid medium to observe for cellulose pellicle formation.

Plating on a selective nutrient medium for mutants. A medium was prepared as follows: 20 g. yeast extract (Difco) were shaken with 200 ml. of a mixture of ethanol and water (4:1) in a separating funnel; the supernatant liquid was decanted and the ethanol removed from it under vacuum (below 40°). Then the residue was made up to a final volume of 40 ml. with water. This solution (1 ml.) + glucose (3 g.) + agar (2 g.), made up to 100 ml. with water, comprised the selective medium (pH 6·2).

The organisms used for plating were obtained from 48 hr. GYE parent cultures, each grown from a different single-colony isolate. Inoculum suspensions were prepared by removing the pellicles and washing them in several changes of sterile water, after which they were finally shaken in 5 ml. of water to remove as many organisms as possible from the pellicle. Volumes (*c.* 1 ml.) of this last suspension were added to each Petri dish and mixed with 15 ml. of selective medium; six replicate plates were prepared with each isolate. Incubation was for 12 days.

Replica plating on the selective medium. The method of Lederberg & Lederberg (1952) was followed.

Mass-plating on GYE medium. This method for the detection of mutants in the parent culture arose from the observations (made during the present study) that when a sufficiently large inoculum from a cellulose-producing culture was spread on the surface of GYE agar, growth of mutant clones could be observed. Mutant colonies have a faster growth rate than parent colonies and also their colony type is different from that of wild-type organisms.

Each culture (in GYE medium) grown from a single-colony isolate, was well shaken to dislodge as many organisms as possible from the cellulose pellicle. Then the desired volume (0·10 or 0·20 ml.) of inoculum, removed aseptically, was spread with a sterile glass rod over the surface of GYE agar and incubated.

RESULTS

Experiments with Acetobacter acetigenum *NCIB* 8132

Static cultures in GYE medium. Over a period of about one year more than 150 cultures were prepared; in no case was the loss of ability to produce cellulose observed.

Static cultures in EYE medium. In 10 experiments which included work with 26 cultures, the following results were obtained: 12 cultures produced cellulose pellicles, 8 gave no visible growth, and 6 produced diffuse growth with no visible cellulose pellicle. The cultures which formed cellulose pellicles grew very slowly in this medium; the pellicles appeared after 3–5 days of incubation. The appearance of diffuse growth generally occurred, if at all, between 24 and 48 hr. of incubation, although in some cases it appeared as late as 5 days. It is apparent from these results that the parent culture did not grow normally in EYE medium, since cellulose pellicles appeared in only 12 of the 26 tests; the production of cellulose by these 12 cultures might be attributed to the carry-over of glucose in the inoculum. After 10 serial subcultures in GYE medium at 72 hr. intervals the turbid cultures still failed to produce cellulose pellicles, whereas the parent culture produced submerged zoogloeal material in 24 hr., followed by a surface cellulose pellicle in 36–48 hr.; the medium below these pellicles remained virtually clear. When the diffuse-growing mutant cultures were plated (GYE agar) colonies were observed which were quite different from those of the parent culture. Parent colonies were punctiform, about 1 mm. in diameter, pale brown in colour, circular, convex, with a regular edge and tough consistency; they appeared on the plates after 3–5 days incubation. In contrast, mutant colonies were 2–4 mm. in diameter, white, circular, raised to convex, with a regular edge and butyrous consistency; they appeared on the plates within 24 hr. of inoculation.

An experiment with ethylene glycol in place of ethanol also showed diffuse growth with no visible cellulose pellicle even after 10 subcultures in GYE medium. No growth was observed in media which contained *n*-propanol in place of ethanol.

Shaken cultures in GYE medium. The morphology of the cellulose bodies formed in the shake cultures was similar to that reported by Schramm & Hestrin (1954); the cellulose appeared as stellate bodies which varied in diameter from 2 to 8 mm. In three experiments which consisted of ten, seven and six serial transfers, respectively, cellulose was produced in all the static and shaken cultures. The lack of agreement between these results and those of Schramm & Hestrin (1954) may have been because different species of Acetobacter were used.

Nevertheless, the shaken cultures were plated out at each transfer and isolates were picked to tubes of GYE liquid medium in order to examine them for cellulose production. Of 905 isolates tested, the colony forms of which appeared identical, only three did not form cellulose pellicles. In these instances the organisms grew diffusely throughout the medium and in 24 hr. produced moderate turbidity with no visible cellulose pellicle. In contrast, the cellulose-forming isolates produced submerged zoogloeal material in 24 hr., followed by a surface pellicle of cellulose at 36–48 hr. When plated, the diffuse cultures gave mutant colonies similar to those described earlier.

Shaken cultures in EYE medium. Two tests gave no visible growth while a third gave diffuse growth but no pellicle formation. When the diffuse culture was plated (GYE medium) the colony type and its growth rate differed from that of the parent culture. Eighty-six colonies were picked and transferred to GYE medium to examine their ability to produce cellulose pellicles. About 70 % of these isolates produced a weak pellicle after 5 days of incubation, and showed a moderate degree of diffuse growth; on transfer to fresh medium they did not form a pellicle but showed diffuse growth. The remaining 30 % continued to produce diffuse growth and did not show pellicle production even after ten serial subcultures.

The origin of celluloseless mutants

In these experiments the loss of ability to produce a cellulose pellicle might have been induced by the specific treatment (shaking, presence of ethanol) or the cultural conditions may have favoured the growth of mutants already present in the parent culture. Therefore experiments were made to examine for the presence of celluloseless mutants in static GYE cultures. The selective medium used in this work allowed the growth of mutants but not of parent organisms. (The addition of phosphate or magnesium ion to the selective medium allowed growth of the parent culture).

Plating on the selective medium for mutants. Suspensions of organisms (see methods) prepared from three parent GYE cultures, were examined for the presence of mutants. The inoculum consisted of *c.* 1·7 ($\pm 0{\cdot}3$) $\times 10^6$ bacteria/plate. The results (Table 1) showed that colonies appeared on 14 of the 18 plates prepared. The average number of colonies/plate was 2·06; accordingly, the

Table 1. *Plating of* Acetobacter acetigenum *NCIB* 8132 *on the selective medium*

	No. of colonies/plate		
Plate no.	Suspension* no. 1	Suspension no. 2	Suspension no. 3
1	1	0	1
2	3	9	1
3	1	12	1
4	1	2	2
5	0	1	2
6	0	1	0
Total	6	25	7
Mean/plate	1·0	4·1	1·1
Over-all mean/plate	2·06	—	—

* Each suspension of organisms was prepared from a culture grown from a single-colony isolate.

estimated ratio of mutant to parent organisms was 1 mutant to $8{\cdot}2 \times 10^5$ parent organisms. The organisms grew rather slowly; the first colonies appeared in about 3 days. All of the 38 colonies which appeared on the plates were transferred to GYE liquid medium to test for cellulose production, and of these 31 produced a pellicle and 7 showed diffuse growth with no visible cellulose pellicle.

Replica plating. Although some fairly good replicas were obtained on the selective medium, three attempts to obtain a celluloseless mutant by this method were unsuccessful.

Mass plating. The results of these tests are given in Table 2. After 24 hr. of incubation *c.* 50 % of the plates showed the growth of some mutants. The age of the culture tested did not appear to influence the results significantly. The majority of plates showed confluent mutant growth which covered about one-half of the area of the plate; after 3–4 days of incubation parent-type colonies appeared.

Table 2. *Results of 'mass-plating'* of Acetobacter acetigenum *NCIB* 8132 *on GYE medium*

Test no	Culture age (days)	No. of plates	No. of plates with mutants
1	12 (0·2)*	12	5
2	5 (0·2)	12	8
3	4 (0·1)	7	3
4	2 (0·1)	6	3
	Total	37	19

* Volume (ml.) of culture tested.

Experiments with Acetobacter *spp. NCIB* 5346 *and NCIB* 7029

The culture of *Acetobacter acetigenum* NCIB 5346 was of particular interest since it contained both parent and mutant organisms when received from the National Collection of Industrial Bacteria. The initial GYE culture prepared from the lyophile tube showed diffuse growth at 24 hr. and no visible cellulose pellicle. After 6 days of incubation a cellulose pellicle was produced. Subsequently, three serial subcultures were made at 6-day intervals. At this time the culture did not give diffuse growth but a surface pellicle of cellulose was present after 48 hr. incubation. When this culture was plated (GYE agar) parent colonies were observed which were quite different from those of the celluloseless mutant.

On several occasions during routine subculture on GYE medium (static incubation) cultures of NCIB 5346 and NCIB 7029 lost their ability to produce cellulose pellicles. For this reason, the stock cultures were kept in malt wort medium thereafter. They appeared to be more stable in this medium, since such cultures always retained the ability to form a pellicle even after prolonged subculture. On other occasions, transfers made from cellulose-producing cultures (GYE medium) to fresh medium showed diffuse growth at first (24 hr.) but later (up to 14 days) showed a cellulose pellicle and a marked diminution in turbidity. In all cases, when the diffuse cultures were plated mutant colonies were observed which differed from those of the parent culture. Mutant cultures grown from single-colony isolates picked from the plates did not produce a cellulose pellicle even after repeated subculture for about one year.

The loss of cellulose-forming ability was also observed with cultures of NCIB 5346 and NCIB 7029 when they were inoculated into EYE medium or peptone medium. The frequency of this occurrence was not studied because these cultures showed mutants in GYE medium, as mentioned above.

DISCUSSION

The frequency of the appearance of these celluloseless mutants has been observed to vary with cultural conditions, species and strain. Loss of ability to produce cellulose was observed with cultures of NCIB 8132 when cultivated under conditions which selected against the growth of the parent organism (EYE liquid medium) but not under conditions which favoured the growth of the parent organism (GYE liquid medium). On the other hand, cultures of two other organisms (NCIB 5346 and NCIB 7029) showed mutants under growth conditions which were favourable for the parent culture.

The mass-plating technique indicated the occurrence of celluloseless mutants in static GYE liquid cultures of NCIB 8132. The fact that the mutant had a faster growth rate than the parent on GYE agar medium allowed its detection 24–48 hr. before parent colonies appeared. However, the physiological relationship between these two different types of organism must be somewhat different in GYE liquid medium since cultures which did not produce cellulose pellicles in that medium were not observed.

The fact that some of the colonies which grew on the selective medium (from which it is inferred that these clones contained mutants) formed cellulose when transferred to GYE liquid medium suggested that these colonies were a mixture of parent and mutant organisms; however, the possibility of reversion cannot be excluded. These results also suggest that the parent has a selective advantage over the mutant in GYE liquid medium. Possibly the parent has a faster growth rate than the mutant in the latter medium, although this was not supported by visual observations of growth.

The demonstration of mutants by the mass-plating technique and the failure to obtain mutants by the method of indirect selection (Lederberg & Lederberg, 1952) are inconsistent. Preliminary studies on this aspect have revealed that the parent culture (in GYE liquid medium) produces a factor which is inhibitory to the growth of mutants. This would appear to offer an explanation for the failure to enrich the population in the liquid medium with the mutants. This is being studied further.

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REFERENCES

Creedy, A. E., Jowett, P. & Walker, T. K. (1954). Formation of D-cellobiose from starch and from other substrates by an *Acetobacter* species. *Chem. & Ind.* (*Rev.*), **73**, 1297.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. bact.* **63**, 399.

Schramm, M. & Hestrin, S. (1954). Factors affecting production of cellulose at the air-liquid interface of a culture of *Acetobacter xylinum*. *J. gen. Microbiol.* **11**, 123.

Tosic, J. & Walker, T. K. (1946). Procedure for the characterisation of the acetic acid bacteria. *Chem. & Ind.* (*Rev.*), **65**, 104.

Wright H. B. & Walker, T. K. (1955). Synthesis of melibiose by an *Acetobacter* species in a lactate-buffered glucose medium. *Chem. & Ind.* (*Rev.*), **74**, 18.

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O'DEA, J. F. & DINEEN, J. K. (1957). *J. gen. Microbiol.* **17**, 19–24

Fluorescent Antibody Studies with Herpes Simplex Virus in Unfixed Preparations of Trypsinized Tissue Cultures

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SUMMARY: The classical strain of herpes simplex virus (Burnet & Lush, 1939) and a recently isolated strain (Melbourne 1/56) were propagated in tissue culture of human amnion and infant-mouse kidney 'epithelial' cells. Following the inoculation of $10^{3\cdot25}$ $TCCD_{50}$ (tissue culture cytopathogenic dose) of the Melbourne 1/56 herpes simplex virus into amnion cell culture, the titre of virus dropped to $10^{0\cdot50}$ $TCCD_{50}$ at 12 hr. and the maximum level of virus ($10^{3\cdot50}$ $TCCD_{50}$) was detected at 48 hr. after inoculation. The earliest morphological changes were observed in the tube-cultures *c.* 18 hr. after inoculation of the virus and eosinophilic intranuclear inclusion bodies were observed in the amnion cells *c.* 24 hr. after virus injection. Cell degeneration was prominent at 36 hr. when most cells were highly refractile and rounded. Cell suspensions, harvested from infected and uninfected cultures of amnion and mouse-kidney epithelium, were treated with immune herpes serum followed by fluorescent rabbit anti-human γ-globulin, and specific peripheral fluorescence was observed in the unfixed preparations of the infected tissue cultures. The limited surface staining was ascribed to the impermeability of the unfixed cytoplasmic membranes to globulins.

Weller & Coons (1954) by using the 'double-layer technique' in fluorescent antibody studies, showed that varicella and herpes zoster viruses propagated in tissue culture of human embryonic skin, muscle and foreskin. These workers used, as a control of the new technique, tissue culture preparations infected with herpes simplex virus. It is the purpose of the present communication to record work on the specific fluorescent staining of herpes simplex virus in tissue cultures. During this work the non-specific fluorescence which we have observed in acetone-fixed preparations was decreased by use of unfixed preparations.

METHODS

Human amnion cell cultures. Single cells and small cell-aggregates were released from the amnions by trypsinization. The cell suspensions were washed once with Earle's saline (Earle, 1943) and resuspended at a concentration of 150,000, single cells and cell-aggregates/ml. in a nutrient medium of 30 % (v/v) heat-inactivated horse serum, 5 % (w/v) lactalbumin hydrolysate and 65 % (v/v) Earle's saline. Penicillin (100 units/ml.) and streptomycin (100 μg./ml.) were added to the complete medium. The pH value of the final cells suspensions was adjusted to the acid side of neutrality (*c.* pH 6·8) with carbon dioxide and samples (1 ml.) were dispensed into roller tubes. After incubation of the cultures at 37° for 3 days in stationary racks, the medium was changed and the cultures set rotating at 40 rev./hr. When luxuriant proliferation of the cells had occurred (about the 7th or 8th day) the medium was again replaced and the cultures were then ready for virus inoculation.

Infant-mouse kidney-cell cultures. The cultures of infant-mouse kidney epithelium were similarly prepared, but more rapid proliferation occurred. The cultures were generally ready for virus inoculation by the 5th day after trypsinization.

Harvesting of tissue cultures. Infected and uninfected sheets of cells were released from the surface of the tubes by adding 1 ml. 0·25 % (w/v) trypsin at 37°. Infected cultures were harvested on the first and second days following virus inoculation. The cell suspensions from each tube were washed twice and re-suspended in 0·2 ml. phosphate buffered saline (pH 7·0) and the cell concentrates so obtained were then ready for treatment with fluorescent antibody.

Herpes simplex virus. The classical HFE strain (Burnet & Lush, 1939) and a strain (Melbourne 1/56) isolated from herpetic vesicle fluid in tissue culture of human amnion cells, were used throughout the present studies. The Melbourne 1/56 and the HFE viruses were maintained through six and two serial amnion cell passages, respectively, before use.

Newcastle disease virus. The strain of NDV isolated by Albiston & Gorrie (1942) was used in the control studies.

Virus titrations. Titration of the Melbourne 1/56 herpes simplex virus was carried out in amnion cell tissue culture at tenfold dilutions with six tubes/dilution, and examined under low-power light microscopy for cytopathogenic effects on the 5th and 7th day after inoculation. Virus titres were estimated by the method of Reed & Muench (1938).

Virus titres were estimated on the supernatant fluid of pools of six amnion cell cultures at intervals following inoculation with 6th-passage material of the Melbourne 1/56 herpes simplex virus. The inoculum (0·05 ml.) contained $10^{3\cdot 25}$ tissue culture cytopathogenic doses $_{50}$($TCCD_{50}$) of virus and specimens were harvested at 12, 24, and 36 hr. and 2, 3 and 5 days.

Preparation of rabbit anti-human γ-globulin. Electrophoretically homogeneous γ-globulin (Commonwealth Serum Laboratories; 2 ml.; 320 mg. protein) was intimately dispersed with sterile 'Arlacel A' (0·25 ml.) by using a 5 ml. syringe and an 18-gauge needle. Sterile 'Bayol F' (1·5 ml.) was slowly incorporated into the mixture and the fine opalescent emulsion so obtained (3·75 ml.) was injected intramuscularly into the thighs of an adult male rabbit. The rabbit was bled from the marginal ear vein 3 weeks after injection and the serum had a titre of 1/25,000 against a one in four dilution of the human γ-globulin in physiological saline when tested by ring precipitation.

The rabbit antiserum yielded a pseudoglobulin fraction when treated with ammonium sulphate (Coons & Kaplan, 1950) or with methanol at 0° (Pillemer & Hutchinson, 1945). Subsequent coupling of this pseudoglobulin fraction with fluorescein isocyanate was essentially as described by Coons & Kaplan, 1950; comparable results were obtained when either aminofluorescein I, aminofluorescein II, or mixtures of these isomers were employed as starting materials. After dialysis at 4°, the coupled antibody (with merthiolate added to 1/10,000) was stored at −15°; immediately before use small portions were absorbed twice with acetone-extracted mouse liver (Coons & Kaplan, 1950).

Human sera. The neutralizing activity of the human sera used in this work was assessed by the inhibition or otherwise of the development of pocks on the chorioallantoic membrane of eggs following the inoculation of 100 egg-infective doses of herpes simplex virus. Several immune and non-immune sera were thus obtained. All human sera were kept at −20° without preservative, and were inactivated at 56° for 30 min. before use.

Staining technique. Preliminary investigations indicated that fixation by acetone or by 95 % (v/v) ethanol in water enhanced the degree of non-specific staining; unfixed cell preparations were therefore used throughout this work.

Infected and uninfected trypsinized 'cell concentrates' (*c.* 0·2 ml.; see *Harvesting of tissue cultures*) were treated separately in small centrifuge tubes with 1·0–1·5 ml. of a 1/10 dilution of herpes-immune human serum in phosphate buffered saline (pH 7·0). After 30 min. with occasional shaking, the cell suspensions were lightly centrifuged at 2500 rev./min. for 3 min. and the supernatant fluids carefully removed. The cell deposits were washed twice within 15 min. with phosphate buffered saline (pH 7·0) and treated with 0·3 ml. of absorbed fluorescein-conjugated rabbit anti-human γ-globulin for 30 min. with occasional shaking. The cell preparations were then centrifuged and washed twice with phosphate buffered saline (pH 7·0), and finally suspended in 0·2 ml. of the same phosphate buffered saline. A drop of the stained cell suspensions was placed on a slide under a coverslip and examined in the fluorescence microscope.

Optical and photographic equipment. The simplified fluorescence microscope described by Matthaei (1950) and Hicks & Matthaei (1955) was used throughout this work, thereby eliminating the need for mercury vapour or arc lamps or for an expensive quartz condenser. The microscope was fitted with an Abbe condenser, a fixed oil condenser of N.A. 1·4, and a decentrable iris diaphragm. A Kodak Wratten G. 15 filter was used in the ocular. Visible blue light for the excitation of fluorescence was obtained by an ammoniacal copper sulphate filter inserted between the light source (a 12 V. 100 W. projection lamp) and the condenser. In using this microscope, it was found that by partly closing the diaphragm and moving it horizontally off centre, a pseudo darkground effect was produced. This effect allowed identical fields to be photographed under darkground or fluorescence conditions. Photographs were taken with an Exacta Varex camera using Kodak Tri X film. Exposure times were 5 min. for fluorescence photographs and $\frac{1}{5}$ to $\frac{1}{25}$ sec. for darkground photographs.

Controls. The following control systems were investigated, using infant-mouse kidney cells and human amnion cells in tissue culture:

(*a*) Uninoculated tissue cells treated with: (i) fluorescent rabbit anti-human γ-globulin; (ii) herpes non-immune human serum followed by fluorescent rabbit anti-human γ-globulin; (iii) herpes immune human serum followed by fluorescent rabbit anti-human γ-globulin.

(*b*) Tissue cells inoculated with herpes simplex virus (one or two days after inoculation and showing obvious cell infection and rounding off) treated with: (i) herpes non-immune human serum followed by fluorescent rabbit anti-human γ-globulin; (ii) herpes immune serum followed by normal rabbit serum conjugated with fluorescein isocyanate.

(*c*) Tissue cells inoculated with Newcastle disease virus (2 days after inoculation and showing cell necrosis) treated with: herpes immune serum followed by fluorescent rabbit anti-human γ-globulin.

RESULTS

Virus growth. Following the inoculation of $10^{3.25}$ $TCCD_{50}$ of the Melbourne 1/56 herpes simplex virus into amnion cell culture, the titre of virus dropped to $10^{0.50}$ $TCCD_{50}$ at 12 hr. and the maximum level of virus ($10^{3.50}$ $TCCD_{50}$) was detected at 48 hr. after inoculation.

Light microscopy. The earliest morphological changes were observed in the tube-cultures *c.* 18 hr. after inoculation of the virus, when the cytoplasm of the cells became more refractile and granular. Characteristic eosinophilic intranuclear inclusion bodies were observed in the amnion cells *c.* 24 hr. after virus injection and cell degeneration was prominent at 36 hr. when most cells were highly refractile, rounded and readily differentiated from the polygonal-shaped uninfected 'epithelial' cells. Occasionally multinucleated syncytial-like masses were observed in infected cultures when degeneration was advanced on the second and third day after virus inoculation. Similar morphological changes were observed in amnion cells infected with the classical HFE strain of herpes simplex virus.

Fluorescence microscopy. No specific yellow-green fluorescence was observed in any of the controls. Apparently the ocular filter (Wratten G15) transmitted some blue light as both infected and uninfected cells were generally visible in vague outline in the fluorescence microscope as a faint blue-grey luminescence rather than a fluorescence. This effect was more obvious in old tissue culture preparations when presumably the proportion of dead cells was high. When infected cells were treated with immune herpes serum, followed by fluorescent rabbit anti-human γ-globulin, the resulting fluorescence was of the kind shown in Pl. 1 and 2, figs. 1–4; darkground photographs of the same cells are included for comparison (Pls. 1 and 2, figs 1*b*–4*b*). Heavily infected cells were readily distinguished from others by the bright peripheral fluorescence which was characteristically yellow-green. Definite intracellular fluorescence was occasionally observed but it was not possible to interpret whether such fluorescence was nuclear or cytoplasmic. With the mouse kidney system the peripheral fluorescence was again marked (Pl. 2, fig. 4), and the over-all cellular fluorescence was more pronounced.

Using the single criterion of peripheral fluorescence in groups of infected cells, eight randomly selected human sera were assayed by the fluorescence technique. Five of these sera gave regular bright fluorescence when applied to infected cells, while the remaining three gave no fluorescence under the same conditions. This result correlated with the neutralization tests in which fluorescence-positive sera were found to inhibit the growth of herpes simplex in eggs, while the fluorescence-negative sera gave no inhibition.

DISCUSSION

The correlation of the results obtained with fluorescence microscopy and those of the neutralization tests shows that the 'double-layer technique' gives unequivocal results in the herpes simplex tissue culture systems described above. Providing that adequate controls are included, the use of the technique of using fluorescent rabbit anti-human γ-globulin eliminates the need for a conjugated specific fluorescent antiserum for each infecting agent. In this work we have included the controls used by other workers as well as a separate control with Newcastle disease virus. No fluorescence was detected in cell preparations stained with the double-layer technique with herpes immune serum, when necrosis was induced by Newcastle disease virus. Thus cell damage by a virus agent serologically unrelated to the immune serum did not, *per se*, give rise to uptake of specific fluorescent material.

In most of our positive preparations, the fluorescence observed was of the peripheral type; this we consider largely due to the use of unfixed preparations. The peripheral or surface staining was attributed to the impermeability of the cytoplasmic membrane to immune- and fluorescein-conjugated globulins. Cytoplasmic or nuclear staining was not convincingly observed, despite the use of 24 hr. infected cultures in which intranuclear inclusions were demonstrated by ordinary light microscopy.

Both fluorescing and non-fluorescing cells were observed in the virus-inoculated tissue cultures. Thus, while some cells were obviously infected and absorbed fluorescent antibody through the specific γ-globulin bridge, virus-infection of the unstained cells cannot be confidently postulated.

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REFERENCES

ALBISTON, H. F. & GORRIE, C. J. R. (1942). Newcastle disease in Victoria. *Aust. vet. J.* **18**, 75.

BURNET, F. M. & LUSH, D. (1939). The inactivation of herpes virus by immune sera: experiments using the chorio-allantoic membrane technique. *J. Path. Bact.* **48**, 275.

COONS, A. H. & KAPLAN, M. H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.* **91**, 1.

EARLE, W. R. (1943). Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. nat. Cancer Inst.* **4**, 165.

HICKS, J. D. & MATTHAEI, E. (1955). Fluorescence in histology. *J. Path. Bact.* **70**, 1.

MATTHAEI, E. (1950). Simplified fluorescence microscopy of tubercle bacilli. *J. gen. Microbiol.* **4**, 393.

PILLEMER, L. & HUTCHINSON, M. C. (1945). Determination of albumin and globulin contents of human serum by methanol precipitation. *J. biol. Chem.* **158**, 299.

Reed, L. J. & Muench, H. (1938). Simple method of estimating fifty per cent end-points. *Amer. J. Hyg.* 27, 493.

Weller, T. H. & Coons, A. H. (1954). Fluorescent antibody studies with agents of varicella and herpes zoster propagated in tissue culture. *Proc. Soc. exp. Biol., N.Y.* **86**, 789.

EXPLANATION OF PLATES

Plate 1

Fig. 1*a*. Human amnion cells infected with herpes simplex (Melb. 1/56) and stained with fluorescent antibody 24 hr. after addition of the virus. (Magnification, × 600.)

Fig. 1*b*. Darkground micrograph of same field shown in Fig. 1*a*.

Fig. 2*a*. Human amnion cells infected with herpes simplex (HFE) and stained with fluorescent antibody 48 hr. after addition of the virus. (Magnification, × 600.)

Fig. 2*b*. Darkground micrograph of same field shown in Fig. 2*a*.

Plate 2

Fig. 3*a*. Human amnion cells infected with herpes simplex (Melb. 1/56) and stained with fluorescent antibody 48 hr. after addition of the virus. (Magnification, × 520.)

Fig. 3*b*. Darkground micrograph of the same field shown in Fig. 3*a*.

Fig. 4*a*. Infant-mouse kidney cells infected with herpes simplex (HFE) and stained with fluorescent antibody 24 hr. after addition of the virus. (Magnification, × 480.)

Fig. 4*b*. Darkground micrograph of the same field shown in Fig. 4*a*.

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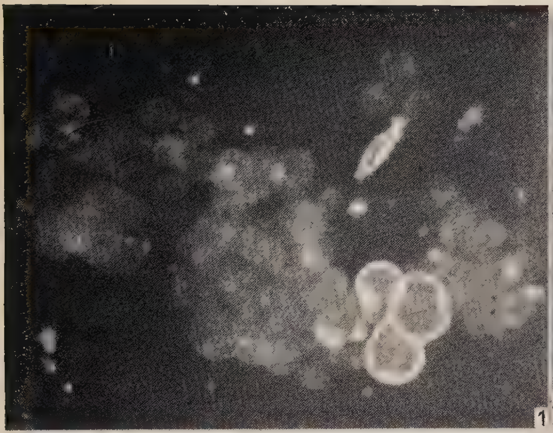

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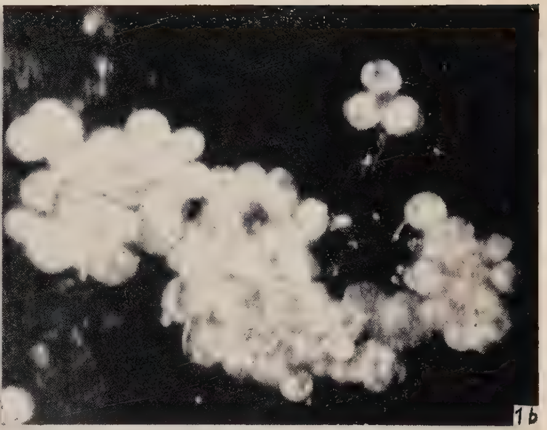

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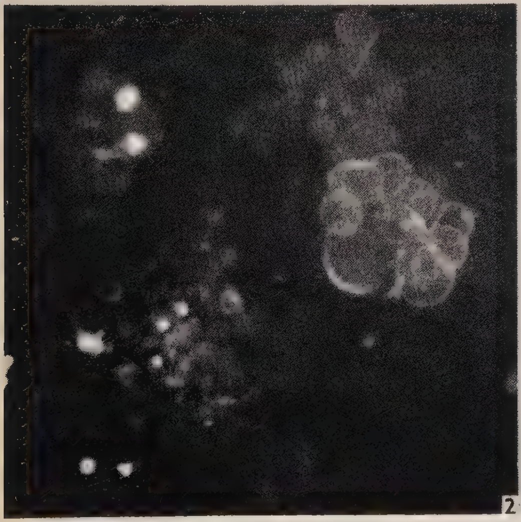

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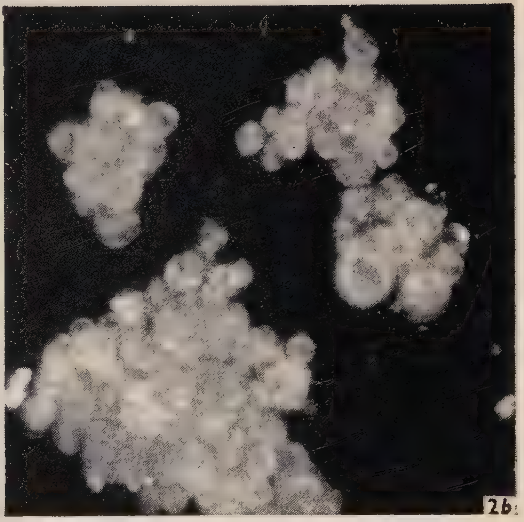

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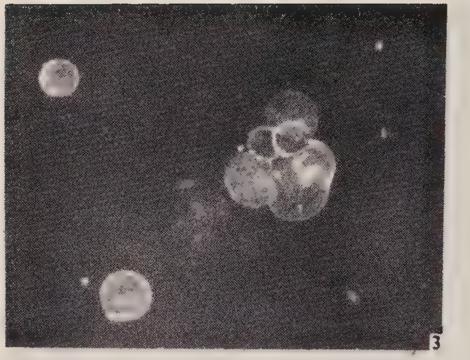
J. F. O'Dea and J. K. Dineen—Fluorescent antibody studies. Plate 1

(*Facing p.* 24)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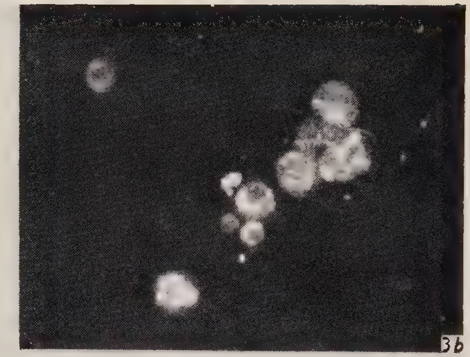
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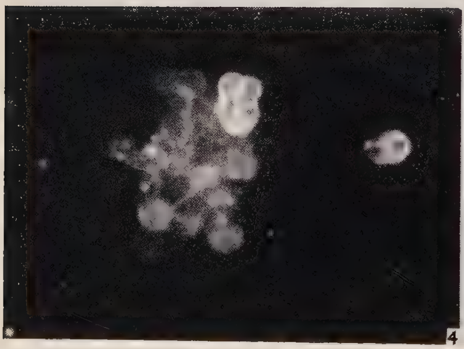
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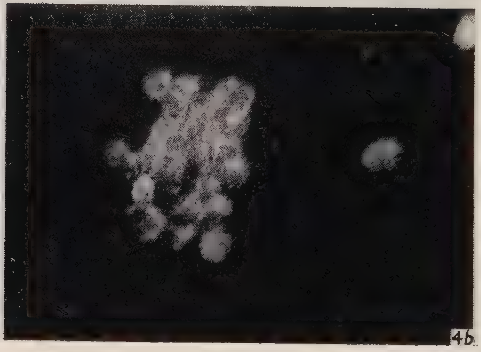
4b

J. F. O'Dea and J. K. Dineen—Fluorescent antibody studies. Plate 2

Edney, M., (1957). *J. gen. Microbiol.* **17**, 25–38

Genetic Analysis of the Development of Serum Resistance in an Influenza Virus Strain

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SUMMARY: During passage of the influenza A strain CAM in eggs in the presence of anti-CAM serum, three true-breeding variants were isolated. The variants I*c*, I*s* and SP_7 showed increasing resistance to inhibition by anti-CAM serum. The evidence indicated that virus population changed in the course of passage as a result of the appearance of successive variants capable of preferential survival in the experimental environment. The strains I*s* and SP_7, unlike the parent strain CAM, were pathogenic for mice inoculated intranasally. The implications of these findings are discussed.

Antigenic variants of influenza virus strains have been produced readily in the laboratory by passing the original strain in the presence of immune serum in eggs (Archetti & Horsfall, 1950; Isaacs & Edney, 1950*b*; Isaacs, Gledhill & Andrewes, 1952; Burnet & Lind, 1954*b*) or in partially immune animals (Magill, 1955; Gerber, Loosli & Hamre, 1955; Gerber, Hamre & Loosli, 1956). The variants reacted poorly with immune serum prepared against the parent strain and retained this property on further passage in the absence of antiserum. It has been generally assumed that the variants arose by spontaneous mutation and selective survival. Few genetic studies of the process of the replacement of original population by variants have been carried out owing to the difficulty of analysing mixed cultures of virus particles which are not antigenically distinct. The use of the limiting dilution technique allows isolation of presumably 'pure clones' of virus particles, the maintenance of a stable virus population and the identification of at least the major component of a virus population (Burnet & Bull, 1943; Isaacs & Edney, 1950*a*; Burnet, 1951).

The present work was begun as an extension of the studies by Burnet & Lind with the A strain CAM. They produced a serum-resistant strain, CAM-SR by carrying out six consecutive passages of CAM in the presence of anti-CAM serum (Burnet & Lind, 1954*b*). Their experiment was repeated in the hopes of isolating a similar resistant strain and of analysing the virus populations at different stages in the series of passages in the serum. It was hoped that characterization of the virus strains isolated from successive passage fluids would throw some light on the process by which serum resistance was developed.

METHODS

Virus strains. CAM, influenza A strain (Anderson & Burnet, 1947); CAM-MP, mouse-pathogenic CAM produced by passing CAM 20 times serially in mouse-lung (Burnet & Lind, 1954*b*); CAM-SR, serum resistant CAM produced by passing CAM six times serially in eggs in the presence of anti-CAM serum (Burnet & Lind, 1954*b*); CAM-MPSR mouse, pathogenic CAM-SR produced by passing CAM-SR twenty times serially in mouse-lung (Burnet & Lind, unpublished).

Haemagglutination (HA) titrations. These titrations were carried out in plastic trays. Virus was diluted in twofold steps in 0·25 ml. volumes of normal saline, an equal volume of a 1 % suspension of fowl red cells was added and the pattern read after standing for 35 min. at room temperature. A standard degree of partial agglutination was taken as the end-point. The smallest amount of virus giving partial agglutination was taken as one agglutinating dose (AD). The HA titre was the reciprocal of the initial dilution of virus at the end-point.

Preparation, absorption and titration of antisera. Immune sera were prepared in rabbits by inoculating intravenously a small dose of virus (1 ml. undiluted allantoic fluid; 2000 AD) and 24 days later a heavy dose of centrifuged concentrated virus (100,000 AD). The rabbits were bled 6 days after the second injection. Sera were inactivated at 56° for 30 min. and stored at −10°.

Sera for absorption were treated for 2 hr. at 37° with RDE, the receptor-destroying enzyme of Burnet & Stone (1947). Heterologous virus was concentrated by centrifugation and added to the serum + RDE mixture to give a final 1/9 dilution of serum. The mixture was left overnight at 4° and then centrifuged at 22,000 g for 1 hr. The supernatant fluid was heated at 65° for 30 min. in the presence of 2 % (w/v) citrate to inactivate RDE and any residual virus, the final dilution of serum being 1/10.

Haemagglutination-inhibition titrations of immune sera (subsequently called anti-HA titrations) were carried out in plastic trays. Serial two-fold dilutions of the sera were prepared in normal saline in 0·25 ml. volumes. One drop of virus containing 5 AD followed by 0·25 ml. of 1 % fowl red cells were added to each cup. After 35 min. standing at room temperature the pattern was read, the anti-HA titre of the serum being taken as the reciprocal of the highest dilution of serum showing partial agglutination.

In estimating the neutralization titres of sera in eggs, the test virus was first titrated for infectivity. Tenfold dilutions of virus were inoculated allantoically into groups of 6 eggs which were incubated for 3 days at 35°. The allantoic fluids were tested for HA and the 50 % infectivity end-point calculated by Thompson's moving average method (Thompson, 1947) with the aid of the Lim nomogram (Lim, 1954). Volumes of virus (0·5 ml.), diluted to contain one thousand 50 % egg-infective doses were added to 0·5 ml. volumes of serial twofold dilutions of serum and 0·05 ml. volumes of the mixtures inoculated into groups of 4 eggs. After 3 days of incubation at 35° the allantoic fluids were tested for HA titre. The reciprocal of the initial dilution of serum at which 50 % of the eggs were infected was taken as the neutralization titre of the serum.

Tests for marker characteristics of virus strains. Heat stability of haemagglutinins, conversion to the indicator state for heat-stable inhibitors and pathogenicity for the chick-embryo were tested by methods frequently described in publications from this laboratory (e.g. Lind & Burnet, 1953).

Titrations of 'Chu' inhibitor present in normal sera were carried out in the same way as anti-HA titrations of immune sera except that fowl red cells were added 30 min. after the test virus.

Mouse-lung pathogenicity (MLP) was measured by inoculating groups of four 5-week-old mice intranasally with 0·05 ml. of freshly harvested preparations of virus diluted to a titre of 1 AD/0·25 ml. Mice were killed 7 days after inoculation and lung lesions recorded according to the arbitrary scale of Fazekas de St Groth & Donnelley (1950). Specific death on the fifth day or earlier was given the score 5·0; death on the sixth day 4·5; 100 % lung consolidation on the 7th day scored 4·0, 75 % 3·0, 50 % 3·0, and 25 % 1·0. The lung lesion score, used as a measure of MLP, was the average for the four mice in the group.

Limiting dilution (*LD*) *passage*). Infectivity titrations were carried out using 6 eggs/tenfold dilution of virus. After 3 days of incubation the allantoic fluids were tested for HA. LD fluids were those harvested after inoculation of the highest dilution of virus giving any positive fluids. Seed viruses of new strains were always second LD fluids, i.e. two LD passages were carried out after isolation.

RESULTS

CAM virus was passed serially in eggs in the presence of anti-CAM serum. After seven passages in serum a virus strain was isolated which was resistant to inhibition by the antiserum and hence distinguishable from the original strain. Each passage-fluid was stored until the end of the experiment and its virus content examined by the limiting dilution technique.

Serial passage of CAM in the presence of anti-CAM rabbit serum

A limiting dilution (LD) fluid of the stock laboratory seed of CAM was used as the starting material. For the first three passages undiluted allantoic fluid virus was mixed with an equal volume of dilutions of anti-CAM serum and the virus-serum mixtures inoculated into groups of 5 eggs. After 3 days of incubation at 35° the allantoic fluids were tested for HA titre. It became obvious during the experiment that the virus was increasing in resistance to serum, so for later passages the virus was diluted and mixed with undiluted serum. After each passage positive eggs from the group where virus had grown in the greatest relative amount of serum were harvested and part of the fluid with the highest HA titre was used for the subsequent passage, part being stored on dry ice. The fluid obtained from the first passage of CAM was labelled SP_1, the next SP_2, etc. Seven such serum passages were carried out and the SP_7 fluid passed twice at LD without serum and a second LD fluid used as the SP_7 seed. The dilutions of virus and serum used for each passage are shown in Table 1.

Table 1. *Passage of CAM in anti-CAM serum*

Passage no.	Virus inoculated	Final dilution of virus inoculated	Final dilution of serum inoculated	Virus harvested
1	Cam	1/2	1/10	SP_1
2	SP_1	1/2	1/3·2	SP_2
3	SP_2	1/2	1/2	SP_3
4	SP_3	1/10	1/2	SP_4
5	SP_4	1/1,000	1/2	SP_5
6	SP_5	1/5,000	1/2	SP_6
7	SP_6	1/10,000	1/2	SP_7

Properties of CAM, SP_7 and CAM-SR

The properties of CAM and SP_7 were investigated in an attempt to define marker characteristics which would enable them to be distinguished from one another. The properties of Burnet & Lind's strain CAM-SR were investigated at the same time. Antisera were prepared in rabbits against SP_7 and CAM-SR. Anti-HA tests were carried out using sera treated with periodate to remove non-specific inhibitor (Burnet & Lind, 1954*a*). The results are shown in Table 2.

Table 2. *Anti-HA titrations of immune rabbit sera*

These figures are the reciprocals of the anti-HA titres of serum tested against 5 AD of virus.

	Serum		
Virus	Anti-CAM	Anti-SP_7	Anti-CAM-SR
CAM	4610	1160	640
SP_7	140	4610	1150
CAM-SR	670	1490	670

It can be seen that both SP_7 and CAM-SR were relatively resistant to inhibition by anti-CAM serum, although they were not serologically identical. These results were confirmed by cross-neutralization tests in eggs. The levels of cross-reaction between CAM and SP_7 were low enough for monospecific anti-CAM and anti-SP_7 sera to be prepared by absorption with the heterologous virus. All new strains isolated in this work were serologically unlike any other strains of influenza used in the laboratory, so the possibility of laboratory contamination was excluded.

CAM, SP_7 and CAM-SR did not differ significantly in ability to grow in the allantoic cavity, in heat stability of HA, or in conversion to the indicator state for heat-stable inhibitors. Examination of infected allantoic fluids by dark-ground microscopy and electron microscopy revealed some filamentous forms in all three strains. None was pathogenic for chick embryos inoculated chorio-allantoically. However, surprising results were obtained when the viruses were inoculated intra-nasally into mice. SP_7 was found to produce about 50 % consolidation of the lungs 7 days after inoculation of 1 AD of virus. As previously demonstrated by Burnet & Lind (1954*b*) CAM and CAM-SR were non-pathogenic.

Chu (1951) showed that influenza strains which were unadapted to mouse lungs were much more readily inhibited by normal serum than adapted strains. The Chu inhibitor in normal rabbit sera was measured against 5 AD of the three viruses, with the results shown in Table 3. Although consistent differences were obtained, these differences were not great enough to distinguish with confidence between the strains. Consequently three tests only were used for identifying CAM and SP_7: inhibition by absorbed anti-CAM or anti-SP_7 sera; intra-nasal inoculation of mice. The reactions of these strains and of CAM-SR in the tests are shown in Table 4. It can be seen that SP_7 and CAM-SR, both obtained by passage of stock CAM in anti-CAM serum and resistant to this serum, are nevertheless readily distinguishable strains.

Table 3. *Inhibition of HA by normal rabbit sera*

These figures are the reciprocals of the inhibitory titres of serum tested against 5 AD of virus.

	Serum no.				
Virus	1	2	3	4	5
CAM	320	160	320	120	120
SP_7	33	60	60	33	33
CAM-SR	72	72	70	60	70

Table 4. *Properties of CAM, SP_7 and CAM-SR*

	Anti-HA titres of absorbed sera		
Virus	Anti-CAM	Anti-SP_7	MLP*
CAM	3000	< 50	0
SP_7	< 50	3000	2·0
CAM-SR	300	1000	0

* MLP = mouse lung pathogenicity. The figures represent the average score of lung lesions of 4 mice 7 days after inoculation of 1 AD of freshly harvested virus.

Analysis of SP_1 to SP_7 fluids

Infectivity titrations of SP_1 to SP_7 fluids which had been stored on dry ice were carried out. Six eggs were used per tenfold dilution and the eggs incubated for 3 days. Controls grown from CAM and SP_7 seeds were always included. Six allantoic fluids positive at the highest dilutions and one from the lowest dilution inoculated were harvested and tested against anti-CAM and anti-SP_7 sera and for mouse lung pathogenicity (MLP). The limiting dilution fluids were stored on dry ice. A typical set of results obtained from passage of SP_2 is given in the following tables. Table 5 shows the results of LD passage of SP_2 and the selection of fluids for testing. Table 6 gives the results obtained in the tests. For assessment of results the percentage of the average anti-HA titre of the serum against the control homologous virus was calculated. A difference of 50 % from the control was considered to be significant. It can be seen that fluids 4, 5 and 7 behaved like CAM and fluids 1, 2, 3 and 6 were intermediate in serological reactions and showed no mouse lung pathogenicity. A second LD pass of fluid 1 was carried out and yielded only intermediate type fluids.

Table 5. *L.D. Passage of* SP_2

Dilution	Egg no. 1	2	3	4	5	6
10^{-9}	$-$	$-$	$-$	$-$	$-$	$-$
10^{-8}	$-$	$-$	$-$	$-$	$-$	$+^{1}$
10^{-7}	$+^{2}$	$+^{3}$	$+^{*}$	$+^{4}$	$+^{5}$	$-$
10^{-6}	$+^{6}$	$-$	$-$	$+$	$+$	$+$
10^{-3}	$+^{7}$	$+$	$+$	$+$	$+$	$+$

$-$ = No HA detectable after 3 days' incubation; $+$ = positive HA at 3 days.
* HA titre < 10.
Superscripts = fluid numbers used in Table 6.

Table 6. *Analysis of fluids yielded by LD passage of* SP_2

Fluid no.	HA titre	Anti-HA titres of sera: Anti-CAM	Anti-SP_7	MLP
1	120	840	420	0
2	70	1280	480	0
3	40	640	320	0
4	850	1900	< 200	0
5	850	3800	< 200	0
6	56	770	230	0
7	240	2700	< 200	n.t.*
CAM control 1	960	2600	< 200	0
CAM control 2	1070	2600	< 200	0
SP_7 control 1	960	< 200	4300	3·2
SP_7 control 2	850	< 200	2900	2·6

* n.t. = not tested.

It may be argued that the intermediate strains represent mixtures of CAM and SP_7. Hirst & Gotlieb (1953) showed that, in a mixture of two antigenically distinct virus strains, only one strain could be detected in an anti-HA titration. Artificial mixtures of influenza A strains MEL and WSN were inhibited by anti-MEL serum, by anti-WSN serum or by neither, never by both. Since the intermediate strains isolated in the present work were inhibited to some extent by both sera it was concluded that they carried both CAM and SP_7 antigens in one virus particle.

In the course of the examination of SP_1–SP_7 fluids, only four types of virus were found, namely CAM (C), SP_7 (S) and two intermediates. The properties of four typical fluids are given in Table 7. The symbols I*c* and I*s* were chosen to represent the CAM-like intermediate and the SP_7-like intermediate. Comparison with Table 4 shows that neither I*c* nor I*s* is identical with CAM-SR.

The results of the analysis of the seven passage fluids are given in Table 8. The limiting dilution fluids, given in black, were stored on dry ice. One of each type from each passage was passed again at LD and all were found to breed true. Since the titrations were necessarily carried out on different days,

Table 7. *Properties of viruses isolated*

		Percentage control anti-HA titre		Neutralization titres			
Virus type	HA titre	Anti-CAM	Anti-SP$_7$	Anti-CAM	NRS*	MLP	Symbol
CAM	430	100	<2	640	2·7	0	C
Intermediate	50	30	10	350	$<1·0$	0	I*c*
Intermediate	380	8	75	68	$<1·0$	2·2	I*s*
SP$_7$	480	<2	100	28	1·0	2·0	S

* NRS = normal rabbit serum heated at 56° for 30 min.

Table 8. *Analysis of yields from LD passages of SP$_1$–SP$_7$*

The symbols C, I*c*, I*s* and S are defined in Table 7. The limiting dilution fluids are in black.

	Virus fluids harvested						
	High dilution fluids						Low dilution
Virus fluid inoculated	1	2	3	4	5	6	7
SP$_1$	**C**	**C**	C	C	C	C	C
SP$_2$	**Ic**	**Ic**	I*c*	C	C	I*c*	C
SP$_3$	**C**	**Ic**	C	C	C	C	C
SP$_4$	**Is**	**Is**	I*s*	I*s*	I*s*	I*s*	I*s*
SP$_5$	**Is**	**Is**	**Is**	**Is**	**Is**	I*s*	I*s*
SP$_6$	**S**	**S**	**S**				S
SP$_7$	**S**	S	S	S	S	S	S

Table 9

	Anti-HA titres of immune sera		Chu inhibitor titres of normal sera			
Source of virus	Anti-CAM	Anti-SP$_7$	Rabbit	Ox	MLP	Virus type
CAM seed	3840	<100	220	1600	0	C
SP$_1$	3200	<100	240	1150	0	C
SP$_2$	1500	490	220	800	0	I*c*
SP$_3$	1200	500	120	1070	0	I*c*
SP$_4$	120	2560	110	770	2·0	I*s*
SP$_5$	240	3900	30	690	2·7	I*s*
SP$_6$	<100	5300	30	210	3·0	S
SP$_7$	<100	3400	40	<100	2·7	S
SP$_7$ seed	<100	3500	70	140	2·5	S

the stored LD fluids were passed again and all were tested in parallel against anti-CAM and anti-SP$_7$ sera, and against normal rabbit and ox sera for assessment of inhibition by Chu inhibitor and for MLP. The results are shown in Table 9.

It can be seen from Tables 8 and 9 that four types of virus were isolated which bred true on further passage. When the properties of the viruses were tested in parallel the serological difference and the differences in MLP were clear-cut and the passages at which changes in these properties occurred were clearly demarcated. A gradual change in inhibition by Chu inhibitor occurred but this could not be correlated directly with mouse lung pathogenicity. It might be concluded only that inhibition by Chu inhibitor depended on some factor other than genetic constitution of the virus strains; this property demands further investigation.

In the following interpretation of the results it was assumed that virus strains which appeared at limiting infective dilution were present in the original fluid as the major infective component. Thus SP_1 consisted of a majority of C types. SP_2 and SP_3 were a nearly equal mixture of C and I*c*. However, except at the highest dilutions, C overgrew I*c*. (It was noted that I*c* types gave consistently lower yields of HA than other strains.) SP_4 and SP_5 yielded only I*s*; SP_6 and SP_7 only S; SP_7 did not change its character after four passages without serum. It seemed reasonable to assume that any virus type which appeared as the major component in the population harvested from an infected egg was initially inoculated into that egg, i.e. that the mutation did not occur in the egg from which the new strain was isolated. On this assumption SP_1 must have contained a small portion of I*c* particles, SP_3 some I*s* and SP_5 some S.

Table 10. *Passage of SP_3 in anti-CAM serum*

	Virus dilution											
Serum dilution	10^{-2}				10^{-3}				10^{-4}			
1/20	–	–	–	–	–	–	–	–	–	–	–	–
1/100	I*s*	–	I*s*	I*s*	–	I*s*	I*s*	–	–	–	–	–
1/1000	–	C	C	–	C	C	C	–	C	C	–	C

– = No HA detectable after 3 day's incubation. I*s*, C, as in Tables 7 and 8.

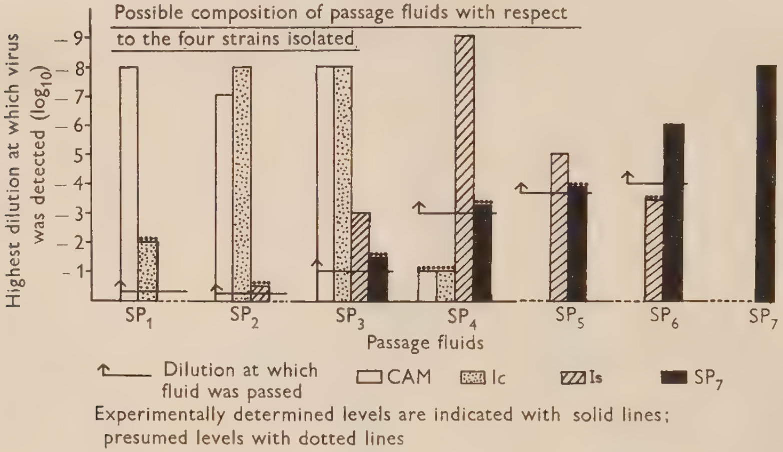

Fig. 1

SP_3 was first analysed further. Mixture of SP_3 and anti-CAM serum in various proportions were inoculated into eggs and all positive fluids were typed; the results are shown in Table 10. I*s* types were isolated when C and I*c* were suppressed by large amounts of anti-CAM serum. Similar experiments were carried out with SP_1 and SP_2. These fluids yielded only CAM. Next,

SP_4 was grown in anti-SP_7 serum to see if any C or I*c* could be detected. SP_4 yielded only I*s* whether passed in anti-CAM serum, anti-SP_7 serum or with no serum.

Fig. 1 has been constructed to show the probable composition of the fluids SP_1–SP_7 with respect to the four strains isolated. It is realized that other virus components which have not been detected experimentally may well have been present. It appears then that the original population of CAM particles has been successively replaced by three mutant strains I*c*, I*s* and SP_7 in various proportions, each strain having a survival advantage over the previous one in the presence of excess anti-CAM serum. Each new strain shows a decrease in CAM antigen and an increase in a dissimilar antigen which has been called SP_7.

Passage of C in anti-CAM serum

C, isolated from limiting dilutions passage of SP_3 (see Table 8) was passed in anti-CAM serum in an experiment similar to that illustrated in Table 1. All positive fluids obtained at each passage were tested for inhibition by anti-CAM and anti-SP_7 sera. In four passages in the antiserum only CAM virus was detected and no increase in serum resistance was observed. The final virus was not pathogenic for mice. This experiment further emphasized the unreproducibility of results in investigations of virus variation.

Passage of CAM-SR and CAM-MPSR in anti-CAM-SR serum

An attempt was made to produce further serum resistance in the strain CAM-SR by passing it in anti-CAM-SR serum. After nine passages in antiserum no obvious change in serum resistance had occurred. The derived virus reacted poorly in anti-HA and neutralization tests with homologous antiserum and with anti-CAM-SR serum. This change appeared to be analogous to the conversion of P to Q virus by passage in antiserum described by Isaacs *et al.* (1952). Similarly, CAM-MPSR, a mouse-pathogenic derivative of CAM-SR, was passed in anti-CAM-SR serum with no change occurring after eleven passages. The derived strain in this case was serologically indistinguishable from the original strain and showed slightly reduced mouse-pathogenicity. The properties of these viruses are shown in Table 11.

Table 11. *Properties of strains derived by passage of CAM-SR and CAM-MPSR in anti-CAM-SR serum*

			Anti-HA titres of sera		
Original strain	Test virus	No. of passages in serum	Original	Derived	MLP
CAM	Original	0	670	1200	0
	Derived	9	190	600	0
CAM-MPSR	Original	0	1500	5000	1·3
	Derived	11	1300	5000	0·9

Investigations of mouse-pathogenicity of Is and SP_7

The pathogenicity for mice of SP_7 and I*s* was compared with that of CAM-MP, a strain derived by passing stock CAM twenty times serially in mouse lung (Burnet & Lind, 1954*b*). Groups of 4 mice were inoculated intranasally with 0·05 ml. volumes of tenfold dilutions of each virus. The first dilution in each case contained 100 AD virus/0·25 ml. so that the 10^{-2} dilution represented the amount usually inoculated for tests of MLP. The mice were killed 7 days later and the lesions examined; the lung lesion scores are shown in Table 12. It can be seen that all three viruses were almost equally pathogenic for mice, although previously CAM-MP had been through twenty passages in mice and SP_7 and I*s* none.

Table 12. *Comparison of mouse-lung pathogenicity of three virus strains derived from CAM*

Dilution of virus inoculated	Lung lesion scores		
	SP_7	I*s*	CAM-MP
1	3·6	4·3	3·8
10^{-1}	2·6	2·9	3·4
10^{-2}	2·1	1·8	2·1
10^{-3}	1·8	1·2	1·3
10^{-4}	0·8	0·6	1·0
10^{-5}	0·1	0·2	0·5
10^{-6}	0·0	0·0	0·0

Table 13. *Protection of mice by CAM and SP_7 vaccines against challenge by SP_7*

Dilution of challenge virus	Lung lesion scores		
	Vaccinated with SP_7	Vaccinated with CAM	Controls
1	0·1	0·9	3·6
10^{-1}	0	1·0	2·6
10^{-2}	0	0	2·1
10^{-3}	0	0	1·8
10^{-4}	0	0	0·8
10^{-5}	0	0	0·1

The immunizing potency of CAM and SP_7 against challenge by SP_7 was investigated. Groups of mice were vaccinated with CAM and SP_7 allantoic fluids heated at 56° for 60 min.; these vaccines were non-infective for chick-embryos. Each mouse received 480 AD of virus inoculated intraperitoneally. The mice were challenged by intranasal inoculation 11 days later with tenfold dilutions of SP_7, each dilution being inoculated into 4 mice vaccinated with CAM, 4 vaccinated with SP_7 and 4 controls. The mice were examined for lung lesions after a further 7 days. The results are given in Table 13. While SP_7 was the better vaccine CAM protected quite well against lung damage by SP_7.

Gerber *et al.* (1956) found that their stock strains PR8 S protected against lethal doses of variants, although the variants reacted poorly with PR8 S antiserum. These authors emphasized the importance of evaluating antigenic changes of influenza viruses with active immunity tests.

DISCUSSION

Before any conclusions can be drawn about variants or mutants which arise during passage of a virus strain in a selective environment is must be reasonably certain that the original virus preparations represented a pure line. Pure lines of bacteriophages are established by two successive replatings from single typical plaques. With certain animal viruses similar techniques may be used in selecting pocks from infected chorio-allantoic membranes or plaques from sheets of fibroplasts (Dulbecco, 1952). For most animal viruses, however, the only technique at present available is the limiting dilution technique, whereby viruses are isolated from a single egg or animal inoculated with dilutions of virus beyond the 50 % infectivity end-point. The applications and limitations of this method were fully discussed by Burnet (1955), who concluded that two successive passages at limiting dilutions were sufficient to give reasonable certainty of pure lines of virus. Few workers in the field of variation in animal viruses have attempted to fulfil this criterion at the beginning of their experiments.

The starting material for the present work was a limiting dilution fluid obtained from the laboratory stock seed of CAM, itself a limiting dilution fluid. Seven serial passages of CAM in anti-CAM serum resulted in the isolation of SP_7, an antigenically-distinct strain resistant to inhibition by anti-CAM serum. Experimental evidence has been presented to show that mutants arose during the passage series and replaced the original strain. Examination of passage fluids by the limiting diluting technique revealed that replacement took place in three stages. After two passages in antiserum the virus harvested represented an almost equal mixture of CAM and a variant I*c*. At the third passage small amounts of a second variant I*s* were detected which was the predominant strain at the fourth and fifth passages. The final strain SP_7 was isolated from the sixth and seventh passage fluids. The three variants maintained their antigenic character on further passage without serum. Each new strain had a survival advantage over the previous one in that it was less readily neutralized by anti-CAM serum.

Stepwise selection of antigenic variants has been demonstrated by Gerber *et al.* (1955) during the passage of the influenza A strain PR8 in partially immune mice. Although the stability and purity of the original virus and the variant strains isolated after a number of such passages were checked by repeated passage in normal animals and by the limiting dilution technique (Gerber *et al.* 1955, 1956) such tests were not carried out on intermediate passage fluids.

The finding that I*s* and SP_7 were pathogenic for mice was most interesting as the parent strain CAM had never been passed in mice and was not pathogenic. The degree of mouse pathogenicity was equivalent to that of CAM-MP, a strain isolated after passage CAM twenty times serially in mouse lung. During studies of adaptation of influenza virus to mice Hirst (1947) found changes in pathogenicity sometimes associated with antigenic changes and sometimes not. Gerber *et al.* (1955, 1956) observed changes in antigenic character of virus during mouse passage not associated with any change in pathogenicity. Two types of experimental approach have led to isolation of mouse-pathogenic variants in eggs without the intervention of mouse-passage, namely recombination experiments (e.g. Perry & Burnet, 1953) and selection of variants by β or Chu inhibitor (Medill-Brown & Briody, 1955). The latter authors isolated influenza strains resistant to inhibition by Chu inhibitor by passing sensitive parent strains in eggs in the presence of excess ox serum, a potent source of the inhibitor. The variants thus isolated were, unlike the parent strains, pathogenic for mice on primary inoculation.

Possible explanations for the acquisition of mouse-pathogenicity by I*s* and SP_7 are selection of mouse-pathogenic strains by the Chu inhibitor remaining in heated rabbit serum, or a chance linkage of the property of mouse virulence with the dominant 'SP_7' antigenic character. The first hypothesis is difficult to test directly, but seems unlikely in view of the facts that normal rabbit serum did not appreciably affect the infectivity of any of the strains, whereas anti-CAM serum was much less active in neutralizing I*s* and SP_7 than CAM and I*c* (see Table 7). It seemed more likely that selection pressure was exerted by the anti-CAM serum and that the property of mouse-pathogenicity was linked genetically to the predominating SP_7 antigen. Presumably the possession of mouse-pathogenicity offered no survival advantage to virus strains multiplying in chick embryos. Such chance mutations to virulence accidentally selected in immune environments might be of great epidemiological importance.

Although experiments on the adaptation of influenza viruses to new environments have given broadly predictable results it is characteristic that details of the changes have never been reproducible. In this laboratory three passages series of CAM in anti-CAM have resulted in the isolation of (*a*) CAM only; (*b*) of I*c*, I*s* and SP_7; (*c*) of CAM-SR. All these strains were serologically distinguishable. Attempts to produce variants from CAM-SR strains resulted in a P–Q type of change or no change at all. The interplay of mutation and selective survival is probably reduced to its simplest form in the present type of experiment. Where there is a more persistent and intense selective process in action variant types may be more readily obtained. It may be noted that Isaacs *et al.* (1952) isolated variants in one passage in eggs when repeated inoculations of antiserum were given during multiplication of the original strain. Variants and 'variants of variants' were readily isolated by passage in immune animals (Gerber *et al.* 1955, 1956; Magill, 1955). Neither of these methods, however, lends itself so well to genetic analysis of the process as simple passage with antiserum in the allantoic cavity.

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REFERENCES

ANDERSON, S. G. & BURNET, F. M. (1947). Sporadic and minor epidemic incidence of influenza A in Victoria 1945–46. 1. Phase behaviour of influenza A strains in relation to epidemic characteristics. *Aust. J. exp. Biol. med. Sci.* **25**, 235.

ARCHETTI, I. & HORSFALL, F. L. (1950). Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. *J. exp. Med.* **92**, 441.

BURNET, F. M. (1951). A genetic approach to variation in influenza viruses. 2. Variation in the strain NWS on allantoic passage. *J. gen. Microbiol.* **5**, 54.

BURNET, F. M. (1955). *Principles of Animal Virology.* New York: Academic Press. p. 394.

BURNET, F. M. & BULL, D. R. (1943). Changes in influenza virus associated with adaptation to passage in chick embryo. *Aust. J. exp. Biol. med. Sci.* **21**, 55.

BURNET, F. M. & LIND, P. E. (1954*a*). Recombination of influenza viruses in the de-embryonated egg. 1. The use of periodate-treated sera for *in vitro* characterization of influenza virus strains. *Aust. J. exp. Biol. med. Sci.* **32**, 145.

BURNET, F. M. & LIND, P. E. (1954*b*). An analysis of the adaptation of an influenza virus to produce lesions in the mouse lung. *Aust. J. exp. Biol. med. Sci.* **32**, 711.

BURNET, F. M. & STONE, J. D. (1947). The receptor-destroying enzyme of *V. cholerae*. *Aust. J. exp. Biol. med. Sci.* **25**, 227.

CHU, C. M. (1951). The action of normal mouse serum on influenza virus. *J. gen. Microbiol.* **5**, 739.

DULBECCO, R. (1952). Production of plaques in monolayer tissue culture by single particles of an animal virus. *Proc. nat. Acad. Sci., Wash.* **38**, 747.

FAZEKAS, de St GROTH S. & DONNELLEY, M. (1950). Studies in experimental immunology of influenza. IV. The protective value of active immunization. *Aust. J. exp. Biol. med. Sci.* **28**, 61.

GERBER, P., LOOSLI, C. G. & HAMRE, D. (1955). Antigenic variants of influenza A virus (PR8 strain). I. Their development during serial passage in the lungs of partially immune mice. *J. exp. Med.* **101**, 627.

GERBER, P., HAMRE, D. & LOOSLI, C. G. (1956). Antigenic variants of influenza A virus (PR8 strain). II. Serological and immunological characteristics of variants derived from variants. *J. exp. Med.* **102**, 413.

HIRST, G. K. (1947). Studies on the mechanism of adaptation of influenza virus to mice. *J. exp. Med.* **86**, 357.

HIRST, G. K. & GOTLIEB, T. (1953). The experimental production of combination forms of virus. I. Occurrence of combination forms after simultaneous inoculation of the allantoic sac with two distinct strains of influenza virus. *J. exp. Med.* **98**, 41.

ISAACS, A. & EDNEY, M. (1950*a*). Variation in laboratory stocks of influenza viruses: biological characters of variants. *Brit. J. exp. Path.* **31**, 196.

ISAACS, A. & EDNEY, M. (1950*b*). Variation in laboratory stocks of influenza viruses: genetic aspects of variations. *Brit. J. exp. Path.* **31**, 209.

ISAACS, A., GLEDHILL, A. W. & ANDREWES, C. H. (1952). Influenza A viruses. Laboratory studies with special references to European outbreak of 1950–51. *Bull. World Hlth Org.* **6**, 287.

LIM, K. A. (1954). A nomogram for 50% end-point interpolation. *J. Hyg., Camb.* **52**, 397.

LIND, P. E. & BURNET, F. M. (1953). Back-recombination of influenza A strains obtained in recombination experiments. *Aust. J. exp. Biol. med. Sci.* **31**, 361.

MAGILL, T. P. (1955). Propagation of influenza virus in 'immune' environments. *J. exp. Med.* **102**, 279.

MEDILL-BROWN, M. & BRIODY, B. A. (1955). Mutation and selection pressure during adaptation of influenza virus to mice. *Virology*, **1**, 301.

PERRY, B. & BURNET, F. M. (1953). Recombination studies with two influenza virus B strains. *Aust. J. exp. Biol. med. Sci.* **31**, 519.

THOMPSON, W. R. (1947). Use of moving average and interpolation to estimate median-effective dose. *Bact. Rev.* **11**, 115.

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BAILEY, L. (1957) *J. gen. Microbiol.* 17, 39–48

The Isolation and Cultural Characteristics of *Streptococcus pluton* and further Observations on *Bacterium eurydice*

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SUMMARY: An account is given of the development of a reliable method for the isolation of *Streptococcus pluton* (*Bacillus pluton* White), an organism associated with European foul-brood disease of the larval honeybee. *S. pluton*, isolated as an anaerobe, may be trained to grow as an aerobe in rod form. Its principal anaerobic growth requirements are a low molar ratio of Na:K, high inorganic phosphate concentration, glucose or fructose, and undetermined factors provided by yeast extract. Peptones are harmful to growth. Aerobic growth has no very critical requirements other than glucose, fructose or sucrose. *Bacterium eurydice* White which, together with *S. pluton*, causes European foul-brood disease grows well anaerobically on a yeast extract + glucose + fructose medium; either sugar alone supports only feeble anaerobic growth. Anaerobic growth of *B. eurydice* is also accelerated by a low molar ratio of Na:K and is inhibited by peptones. *S. pluton* and *B. eurydice* appear to be separate organisms; no evidence has been obtained to support claims by previous workers that *S. pluton* is a variant of *B. eurydice*.

It has recently been established (Bailey, 1956) that *Streptococcus pluton* (*Bacillus pluton* White) is a distinct organism which, together with *Bacterium eurydice* White, causes European foul-brood disease of the larval honeybee. Other bacteria commonly associated with diseased or dead larvae are *Streptococcus liquefaciens* Sternberg (*S. apis* Maassen) and *Bacillus alvei* Cheshire & Cheyne. All four bacteria, either separately or in combination, have at one time or another been considered the cause of the disease. *Streptococcus pluton* has also been thought to be a variant of *Bacterium eurydice* (Burri, 1943) or of *Bacillus alvei* (Lochhead, 1928). A summary of these previous views has been given by N. R. Smith in *Bergey's Manual* (1948) and by Steinhaus (1949). The following is an account of the isolation of *S. pluton* and of some of its cultural characteristics, together with some new observations on *Bacterium eurydice*. The relationships between the cultural characters of these two organisms and their development in the honeybee larva are discussed.

METHODS

The diseased larvae were obtained from colonies of honeybees which were affected to various degrees with European foul-brood disease. The colonies had been kept at an isolated site near Rothamsted Experimental Station, Harpenden, since May 1954. The disease has occurred annually in all these bee colonies and has been allowed to run its normal course without remedial treatment. The symptoms and general nature of European foul-brood disease described fully by White (1920) were found identical with those observed in the infected colonies studied at Rothamsted.

Larvae, from which isolates were to be prepared, were selected carefully. For the attempts in 1954, larvae were chosen which appeared to be healthy but which were surrounded by others in all stages of the disease. In this way it was hoped that the chances of obtaining pure cultures of *Streptococcus pluton* would be increased and that other bacteria to be found in moribund larvae would be avoided. For the subsequent attempts larvae which were showing initial symptoms of the disease were selected; they were slightly displaced in their honeycomb cells or appeared faintly yellow in bright light; but otherwise they had the turgid and glistening appearance of healthy larvae.

The larvae selected were dissected on sterile microscope slides. The cuticle was grasped half way down the length of the body with two pairs of sterile forceps which were then drawn apart. In this way the cuticle was removed, leaving the mid-gut in a pool of body fluid on the slide. A loop of the mid-gut contents was streaked on a plate of the medium. Except where indicated, cultures were incubated anaerobically at *c.* 33° in McIntosh & Fildes jars which contained hydrogen 95 % (v/v) + 5 % (v/v) CO_2. B.D.H. capillator indicators (British Drug Houses Ltd.) were used to determine the pH values of fluids.

RESULTS

The isolation of Streptococcus pluton

Isolates of an oval Gram-positive coccus which seemed likely to be *Streptococcus pluton* were first made on glucose nutrient agar in 1954 by Professor L. P. Garrod (St Bartholomew's Hospital), who was collaborating in the work at this time (*Rep. Roth. Exp. Sta.* 1954); but the growth of this organism was very feeble and it often appeared to degenerate into a variety of pleomorphic forms. It appeared to be an anaerobe, and yeast extract was beneficial to growth. Several attempts were made to infect small colonies of bees with cultures prepared by Professor Garrod, but the disease did not appear. On one occasion, however, the mid-gut of one larva was seen to contain clumps of Gram-positive cocci. Their appearance seemed identical with that of *S. pluton* in naturally-infected larvae. The healthy bees had come from apiaries known to have been free from the disease since 1946 and it was considered very likely therefore that Professor Garrod's isolate was *S. pluton*, but that cultural conditions were unsatisfactory and that virulence had probably been lost. Attempts to repeat or improve the tests in the spring of 1955 were unsuccessful. Efforts were made in September 1955 to re-isolate the organism on different media. The first medium tried was modified from one which had been found suitable for the isolation of *Bacillus larvae* White, which causes American foul-brood of the honeybee larvae. It was made up as follows: 10 g. soluble starch; 10 g. glucose; 5 g. Neopeptone (Difco); 5 g. yeast extract (Difco); 1·36 g. KH_2PO_4; 20 g. agar; diluted to 1000 ml. with distilled water. The pH value was adjusted to 6·8 with concentrated KOH and the medium was sterilized by steaming at 100° for 20 min. on 3 successive days. It will be referred to below as medium I. After one day of incubation there was a slight growth, at the start of the streak, of confluent transparent colonies composed of short rods. After 6 days white colonies about 0·25 mm. diameter were observed to be growing on the confluent colonies. The white colonies were composed of lanceolate streptococci in chains of various lengths. These organisms were Gram-positive and seemed morphologically identical with *S. pluton*. Attempts to subculture these bacteria met with variable success and usually failed on agar plates of medium I; cultures in liquid medium I were more reliable, although growth was only slight. There was an initial growth of thin rods followed by the development of streptococci after about 2 days; acid was formed and the pH value consistently fell to 4·8. At this stage it was believed that the rods were an early phase of the streptococci, as growth of the latter occurred only on the colonies of the rods on agar of medium I and the rods resembled the pleomorphic forms which Professor Garrod had previously isolated. However, since acid was formed, it seems more likely that the rods were *Bacterium eurydice* (see below). Only one more isolate of the streptococcus, together with the thin rods, was made in 1955. More isolates could not be prepared after the middle of September as the disease had died out, as is usual each year at this time.

Attempts to improve the medium were begun; tests were done with subcultures from the two isolates made in 1955, described above. It was found that a tenfold increase of potassium phosphate resulted in a heavy growth of the streptococci and that starch was unnecessary.

For the rest of the winter period, before new isolates could be made again in the spring of 1956, the following medium was used: 10 g. glucose; 5 g. Neopeptone (Difco); 5 g. yeast extract (Difco); 13·6 g. KH_2PO_4; 20 g. agar; diluted to 1000 ml. with distilled water. The pH value was adjusted to 6·8 and sterilization was by steaming as before. This medium will be referred to below as medium II.

Growth of the streptococcus on agar of medium II was reliable and was in pure culture; the rods had been lost.

Home-made yeast extract, equivalent in growth effect to the Difco product, was prepared in two ways:

(1) 100 g. of baker's yeast were added to 450 g. water and then steamed for 1 hr.; after allowing solids to settle the supernatant fluid was decanted and filtered. The solid content of the extract was about 1·2 % (w/v).

(2) 100 g. baker's yeast was allowed to autolyse at 50° for 24 hr. or until liquid, and then diluted with water to 400 ml. After steaming for 30 min. the solid matter was allowed to settle and the supernatant fluid decanted and filtered; the solid content of the extract was about 3·5 % (w/v).

The following peptones were tried instead of Neopeptone (Difco): peptone, Tryptone, Tryptose, Proteose-peptone (Bacto); peptone, Tryptone (Oxoid). None of these improved growth and Bacto-peptone was distinctly inhibitory. Some growth occurred when Neopeptone was entirely replaced by yeast extract but the growth seemed feebler.

When the disease reappeared in the spring of 1956 attempts were made to isolate *Streptococcus pluton* on medium II. A growth of rods of various sizes was obtained, some of which were probably *Bacterium eurydice* and the rest pleomorphic forms of *Streptococcus pluton*, but it was not found possible to isolate the streptococcal form. As *S. pluton* had grown on agar of medium II without Neopeptone further attempts were made with this simpler medium. All attempts were now successful, and two distinct colony forms were always isolated from diseased larvae. The first appeared after incubation for 24 hr. as transparent bluish grey colonies, light brown by transmitted light and composed of thin rods. These were identified as *B. eurydice*, the cultural details agreeing with those given by Gubler (1954). Only feeble growth occurred. Dense white colonies of *S. pluton* became visible after about 2 days. It was not found possible to isolate *S. pluton* on medium II when it contained any kind of peptone. *S. pluton* could be trained to grow in the presence of Neopeptone and it then grew a little more profusely, but it seemed preferable not to use this peptone. The following medium was therefore used: 10 g. glucose, 10 g. yeast extract (Difco), 13·6 g. KH_2PO_4, 20 g. agar, made up to 1000 ml. with distilled water. The pH value of this medium was adjusted to pH 6·6 with concentrated KOH and it was sterilized by steaming. It will be referred to as medium III.

Cultural characteristics of Streptococcus pluton

The cultural characters of the streptococci isolated in 1955 and 1956 from diseased larvae were determined by the use of media II or III as indicated below. Other than the unsuitability of medium II for the initial isolation of *Streptococcus pluton* no results were obtained with it which conflicted with those obtained later with medium III.

Colony characteristics. Growth on agar (media II and III) produced round raised colonies which were 1 or 1·5 mm. in diameter, granular and pearly white by reflected light, and nearly opaque and brownish by transmitted light.

Growth in liquid media. A flocculent growth which appeared at the bottom of the tube (media II and III) about 2 days after inoculation settled to give a dense white deposit of streptococci.

Acid formation. The formation of acid was not detectable (media II and III).

Carbohydrate dissimilation. The following carbohydrates were used: glucose, fructose, sucrose, galactose, lactose, maltose, raffinose, rhamnose, mannitol, sorbitol, inositol, d-xylose, glycerol and starch. Only glucose and fructose supported growth (medium II).

Optimum temperature. Growth, which occurred at 20°, 25°, 30°, 35°, 40°, and 45°, was best at 35° (medium II).

Optimum pH *value.* Best growth occurred at pH 6·5–6·6; reasonable growth occurred between pH 6·0–7·0; hardly any growth was observed at pH 5·5, even after prolonged incubation (medium III).

Effect of CO_2. The presence of CO_2 in the gas phase in concentrations from 1 to 100% (v/v) markedly stimulated growth; the concentration of CO_2 did not seem to be critical (medium II).

Effect of the ratio of Na:K. No growth occurred when the potassium phosphate was replaced with an equimolar concentration of sodium phosphate. Until the molar ratio of Na:K was 1 or less severe inhibition of growth was obvious (medium II).

Phosphate requirement. Decrease of the concentration of potassium phosphate by half usually made no difference to growth, but occasionally it was feebler. Further decrease in phosphate concentration markedly diminished growth (media II and III).

Effect of citrate. Potassium citrate buffer (0·01 M and 0·1 M) was tried instead of phosphate; no growth occurred. Addition of potassium phosphate did not restore growth; but the use of magnesium citrate instead of potassium citrate restored growth when phosphate was present (medium III).

Effect of different sterilization techniques. Growth occurred after medium III had been autoclaved at 5 lb./sq.in. (108°) or 15 lb./sq.in. (121°), but it often seemed inferior to that on the steamed medium; a freshly prepared steamed medium was best. Heat-sterilized media with the sugar added afterwards often did not support any growth. Media which had been sterilized by filtration through a Seitz filter or sintered glass did not support any growth (medium III).

Effect of oxygen. During the development of medium II growth sometimes occurred in broths of this medium enclosed with air in screw-capped bottles. The initial growth was of rods which seemed larger than before and more abundant. No acid was formed; at first this was attributed to the high concentration of phosphate buffer. However, the rods seemed to degenerate when *Streptococcus pluton* began to appear; they were not the same as before, i.e. not *Bacterium eurydice* but were *S. pluton* which had been trained to grow at high oxygen tensions; *B. eurydice* had been lost. In anaerobic culture the streptococci were now the first and only forms which appeared (medium II). It was found later that *S. pluton* could be trained to grow in high oxygen tensions fairly regularly by subcultivation in broths of medium III enclosed with air in screw-capped bottles. It sometimes took 7 days or more before an inoculum from an anaerobic culture began to grow, but it would then grow luxuriantly in further subculture as an aerobe in rod form.

A luxuriant aerobic growth of these rods was obtained on agar (media II and III). The colonies were flat, up to 3 mm. in diameter, fawn-grey by reflected light, and granular and light brown by transmitted light. They had a slightly undulate edge and surface and were composed of Gram-variable rods of varying thickness and length (Pl. 1, fig. 4). The colonies emulsified easily in water, unlike those of *Bacterium eurydice* which are very water-repellant. When grown on agar (media II and III) enclosed with air in a screw-capped bottle the colonies gradually grew transparent and collapsed. As they dwindled, white pin-point colonies appeared which grew to about 1 mm. in diameter; these were colonies of the streptococcal form. The aerobically-trained form subcultured anaerobically in the streptococcal form provided it had been converted into the anaerobic form in each subculture; when it was subcultured under fully aerobic conditions the ability to grow anaerobically was lost, sometimes even after the first subculture. When the streptococcal form from an initially aerobic culture was repeatedly subcultured anaerobically it lost the ability to grow aerobically (usually after two or three subcultures) but could be retrained to do so (medium III). The aerobic form produced an atmosphere rich in CO_2; this was shown by drawing the atmosphere from a culture grown in a screw-capped bottle through lime-water, which rapidly became very turbid (media II and III). No acid was formed (media II and III). The aerobic form had no very critical nutrient requirements; citrate did not inhibit growth nor did excess of sodium over potassium (media II and III). Ordinary laboratory media supported growth provided glucose, fructose or sucrose were available. It was not found possible to cultivate the aerobic form directly from infected larvae (media II and III).

Streptococcus pluton which had not been trained to grow aerobically would nevertheless grow well in atmospheres containing 0·4 % (v/v) O_2, as found by incubating subcultures in desiccators which had been evacuated to the necessary pressure and then filled with hydrogen containing 5 % (v/v) CO_2; but distinct inhibition of growth occurred in 1 % (v/v) O_2 and cocci were poorly differentiated in the chains, which became rod-like (medium III) (Pl. 1, fig. 3).

Catalase production. Aerobic cultures had a high catalase activity. Tubes containing slopes of aerobic cultures were filled with hydrogen peroxide (20 vol.) and inverted over water; the tubes were completely filled with oxygen within a few seconds. Anaerobic cultures, tested in the same way, showed no catalase activity (medium III).

Staining properties. The organisms were usually Gram-positive, but a variable proportion of Gram-negative organisms were always present (media II and III). Aerobic cultures which had converted to streptococcal (anaerobic) forms in closed bottles usually produced clusters of Gram-positive cocci set in a matrix of Gram-negative material; their appearance then resembled most closely that of *Streptococcus pluton* from infected larvae (media II and III). The cocci, which were highly refractile in watery suspension, had thick cell walls which stained well by Robinow's tannic acid + crystal violet method (medium II) (Pl. 1, fig. 6). This has been shown in *S. pluton* from infected larvae (Gubler, 1954).

Maintenance of viability. Cultures kept in an anaerobic atmosphere at room temperatures on agar slopes (medium II) subcultured readily after 6 months.

Cultural characteristics of Bacterium eurydice

These were all determined on Medium III, using as stock cultures organisms isolated from diseased larvae in 1956 on medium III.

Growth on agar. Colonies were transparent, bluish-grey by reflected light and light brown by transmitted light. As described by Gubler (1954) they did not emulsify readily in water. The rods were variable morphologically; often semicircular in early culture they usually developed as straight rods of varying lengths and were about half as thick as *Streptococcus pluton*; there were occasional chains of cocco-bacillary forms.

A second distinct strain was isolated from diseased larvae on medium III; it had rods about as thick as *Streptococcus pluton* and grew in colonies which were denser than those of the other strain of *Bacterium eurydice* but in all the other cultural details listed below the two strains were identical. The occasional chains of cocco-bacilli sometimes bore a superficial resemblance to *S. pluton*, but there was never any evidence of dissociation of these cultures of *B. eurydice* into a form which could be grown with the cultural characteristics of *S. pluton*.

Acid formation. The formation of acid by *Bacterium eurydice* has already been described (Gubler, 1954). In this work the growth of *B. eurydice* decreased the pH value constantly to 4·8, whether growth occurred aerobically or anaerobically.

Effect of glucose and fructose. Glucose or fructose seemed essential for good growth of *Bacterium eurydice* (Gubler, 1954). During experiments upon the effect of sterilizing agents upon mixed cultures of *B. eurydice* and *Streptococcus pluton* in suspension in honey it was seen that a very vigorous growth of *B. eurydice* occurred anaerobically; *S. pluton* did not grow. Further investigation showed that the vigorous growth was caused by the honey unavoidably included in the medium when the suspensions had been plated. It was then found that vigorous anaerobic growth of *B. eurydice* occurred when glucose + fructose were included in the medium; either sugar separately had no stimulating effect at any concentration. The pH value of the medium which contained glucose + fructose rapidly fell to about 4·2 when supporting a culture of *B. eurydice*; it was difficult, for this reason, to obtain growth of *S. pluton* from a mixed culture. (The growth of pure cultures of *S. pluton* is the same on media containing glucose or fructose or both).

Effect of citrate. Aerobic growth of *Bacterium eurydice* was not affected, but anaerobic growth was completely inhibited, by 1 % (w/v) potassium citrate. The equivalent concentration of magnesium citrate did not inhibit growth.

Effect of the molar ratio of Na:K. Anaerobic growth was delayed by about 24 hr. when potassium phosphate was replaced in the medium by an equimolar concentration of sodium phosphate.

Effect of peptones. Anaerobic growth was slightly inhibited by peptones.

Effect of sterilization techniques. Anaerobic growth was delayed in media which had been autoclaved at 15 lb./sq.in. (121°); it was depressed in Seitz-filtered media.

Catalase activity. Aerobic cultures of *Bacterium eurydice* slowly decomposed hydrogen peroxide; anaerobic cultures did not.

Maintenance of viability. Cultures of *Bacterium eurydice* on agar or in broth kept at room temperature, lost viability after about three weeks.

Growth of Streptococcus pluton *and* Bacterium eurydice *in mixed culture*

The two organisms readily grew together (Pl. 1, fig. 2), and the colonies of *Streptococcus pluton* were then often seen growing within the more transparent colonies of *Bacterium eurydice*. When mixed cultures were grown enclosed with air in sealed bottles the initial growth of *B. eurydice* rapidly exhausted the oxygen sufficiently to permit growth of *S. pluton*. Both organisms also grew in mixed culture anaerobically, but then the growth of *B. eurydice* was feeble.

Mixed cultures were freeze-dried; both organisms subcultured readily from the dried preparations after 17 weeks.

DISCUSSION

Some ability to grow aerobically seems to be a normal faculty of *Streptococcus pluton*; the luxuriant aerobic growth which was obtained was probably the result of exaggeration of this faculty by training. A slight catalase activity by the anaerobic form would destroy a small amount of hydrogen peroxide which may be formed in very low oxygen tensions; but growth could be inhibited by excessive formation of hydrogen peroxide in aerobic conditions. This inhibition could be overcome by the development of high catalase activity which occurs when the organism is trained to high oxygen tensions. The lumen of the gut of the healthy larva is perhaps not completely anaerobic; but the oxygen tension may be low enough to allow growth of *S. pluton* in coccal form and apparently in pure culture. White (1912) described and illustrated the appearance of the mid-gut at an early stage of infection, and stated: 'This parasite (*S. pluton*) very early takes a position along the peritrophic membrane and just central to it. At this stage of its growth this micro-organism presents in general an appearance of being rod-shaped with a strong tendency to grow in chains.' The initial rod-like appearance in early infection was also noted by Tarr (1937). It seems likely that a rod-like form of *S. pluton* occurs initially at low oxygen tensions but quickly makes the lumen of the gut sufficiently anaerobic to enable the coccal form to develop.

The inhibition of growth of anaerobic cultures of *Streptococcus pluton* and *Bacterium eurydice* by citrate or by a high molar ratio of Na:K seems to be a feature of lactobacilli (MacLeod & Snell, 1947, 1948). The effect of sodium is unusually severe on *S. pluton*, however; MacLeod & Snell observed half maximum growth of various lactobacilli when the molar ratio of Na:K was 0·81, but inhibition of *S. pluton* is greater than this when the molar ratio is 3 or less. The molar ratio of Na:K in larval blood is about 0·24 (Bishop, Briggs & Ronzoni, 1925) and in honey it is about 0·26 (Wills, 1956); it is likely that the ratio in the gut of the larva is similar.

The high concentration of phosphate in medium III makes easier the initial isolation of *Streptococcus pluton* from diseased larvae; it prevents rapid acidification of the medium by *Bacterium eurydice*. Many difficulties encountered in previous attempts to isolate *S. pluton* were probably largely due to acid production by *B. eurydice*. The success of the isolation by Professor Garrod, of the anaerobic coccus, which was almost certainly *S. pluton*, on glucose nutrient agar, was probably due in part to the selection of apparently healthy larvae which were often infected with almost pure cultures of this organism.

The vigorous anaerobic growth of *Bacterium eurydice* in media which contained fructose + glucose suggests that fructose and glucose are used by different metabolic pathways. It may explain the good growth of *B. eurydice* within the larval gut which, although anaerobic or nearly so when it is supporting a growth of *Streptococcus pluton* in coccal form, must contain fructose and glucose from the honey which forms part of the larval food.

The inhibition of growth of *Streptococcus pluton* in pure culture when the pH value falls below 6·0 and the apparent lack of acid formation suggests that this organism's metabolism is closely adjusted to that of the larva. *Bacterium eurydice*, however, grows quickly and produces an appreciable quantity of acid with which the larva is probably unable to deal. The pH value of the mid-gut contents of moribund larvae is *c.* 4·0; it is about 6·3 in healthy larvae or in larvae which are not showing symptoms of disease but which are supporting a rich growth of *S. pluton*.

It seems likely that *Bacterium eurydice* may be considered the immediate cause of European foul-brood disease but this organism seems able to develop only in larvae which are already infected by *Streptococcus pluton*. Naturally infected larvae often contain a rich growth of *S. pluton*, apparently in pure culture, before symptoms of European foul-brood disease become apparent. White (1912) pointed out that *B. eurydice* is found in comparatively small numbers at an early stage of the disease and then rapidly increases in number; he found that *S. pluton* was always present first in infected larvae. In larvae from bee colonies infected with pure cultures of *S. pluton* there was an initial growth of this organism but the disease did not appear (Bailey, 1956). When attempts were made to infect bee colonies with pure cultures of *B. eurydice* there seemed to be no growth of these organisms at all.

The contents of the gut of the living larva must resist the growth of many organisms which are swallowed; *Streptococcus pluton* and *Bacterium eurydice*, to a lesser degree, must overcome this resistance. *S. pluton* has particularly critical nutritional requirements in anaerobic culture, and these may be linked with a very close adaptation to the environment in the larval gut. *B. eurydice* has some anaerobic nutritional requirements which are similar to those of *S. pluton*, but they are less critical and this may correspond with the lesser degree of adaptation which *B. eurydice* appears to have developed. It seems possible, therefore, that *S. pluton* decreases the resistance of the gut sufficiently to permit growth of *B. eurydice*. Further lowering of the resistance of the contents of the gut to bacterial growth by the development of *S. pluton* and *B. eurydice* was indicated when occasional living larvae, with initial symptoms of disease, were found to contain appreciable numbers of *S. liquefaciens* together with *S. pluton* and *B. eurydice*. All three bacteria were isolated from such larvae. Pure cultures of *S. liquefaciens* fed to healthy larvae did not develop but disappeared. The only other bacterium, commonly associated with European foul-brood disease, which was isolated and identified during this work, was *Bacillus alvei*. This bacillus was found in the remains of dead larvae only, as might be expected, since it is a strict aerobe.

The opinion of Burri (1943) that *Bacterium eurydice* changes into *Streptococcus pluton*, under unknown conditions, seems untenable. Nevertheless, Burri may have succeeded in cultivating *S. pluton*; it may have begun to grow in his initial isolate of *B. eurydice*, in which he was convinced he was growing *S. pluton*, although he was unable to develop subcultures. It seems possible that *B. eurydice* provides nutrients which are utilized by *S. pluton* in an otherwise unsatisfactory medium; the consumption of oxygen by *B. eurydice* might be sufficient, even in the aerobic conditions in which Burri incubated his cultures, to enable *S. pluton* to grow slightly within its colonies. The initial isolation of *S. pluton* on medium I, described above, may have been similar to that obtained by Burri, but under more suitable conditions. Such relationships between the two organisms do not seem possible in the larval gut however, as their sequence of appearance is then the reverse of that in artificial culture.

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REFERENCES

Bailey, L. (1956). Aetiology of European foul brood; a disease of the larval honeybee. *Nature, Lond.* **178**, 1130.

Bergey's Manual of Determinative Bacteriology (1948). 6th ed. p. 724. Edited by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. London: Baillière, Tindall & Cox.

Bishop, G. H., Briggs, A. P. & Ronzoni, E. (1925). Body fluids of the honeybee larva. II. Chemical constituents of the blood and their osmotic effects. *J. biol. Chem.* **66**, 77.

Burri, R. (1943). Weitere Beobachtungen über Formwandlungen beim Erreger der Sauerbrut der Bienen. *Beih. Schweiz. Bienenztg.* **1**, 209.

Gubler, H. U. (1954). Bakteriologische Untersuchungen über die gutartige Faulbrut der Honigbiene (*Apis mellifica* L.). *Schweiz. Z. allg. Path.* **17**, 507.

Lochhead, A. G. (1928). The etiology of European foul brood of bees. *Science*, **67**, 159.

MacLeod, R. A. & Snell, E. E. (1947). Some mineral requirements of the lactic acid bacteria. *J. biol. Chem.* **170**, 351.

MacLeod, R. A. & Snell, E. E. (1948). The effect of related ions on the potassium requirements of lactic acid bacteria. *J. biol. Chem.* **176**, 39.

Report of the Rothamsted Experimental Station (1954). p. 136.

Steinhaus, E. A. (1949). *Principles of Insect Pathology*, 1st ed. p. 244. New York: McGraw-Hill.

Tarr, H. L. A. (1937). Studies on European foul brood of bees. III. Further experiments on the production of the disease. *Ann. appl. Biol.* **24**, 614.

White, G. F. (1912). The cause of European foul brood. *Dep. Circ. U.S. Dep. Agric.* no. 57.

White, G. F. (1920). European foul brood. *Dep. Bull. U.S. Dep. Agric.* no. 810.

Wills, A. P. (1956). Sodium and potassium in Australian foods. *Aust. J. exp. Biol. med. Sci.* **34**, 165.

EXPLANATION OF PLATE

Fig. 1. *Streptococcus pluton* and *Bacterium eurydice* from the mid-gut of a living honeybee larva which had symptoms of European foul-brood disease. × 2000.

Fig. 2. *S. pluton* and *B. eurydice*, from a mixed anaerobic culture. × 2000.

Fig. 3. *S. pluton*, cultured on agar in an atmosphere containing 1 % (v/v) oxygen. × 2000.

Fig. 4. *S. pluton*, trained to aerobic growth and cultured aerobically on agar. × 2000.

Fig. 5. *S. pluton*, trained to aerobic growth and cultured in agitated broth. × 2000.

Fig. 6. *S. pluton*, from anaerobic culture with cell wall stained by Robinow's tannic acid + crystal violet method. × 2000.

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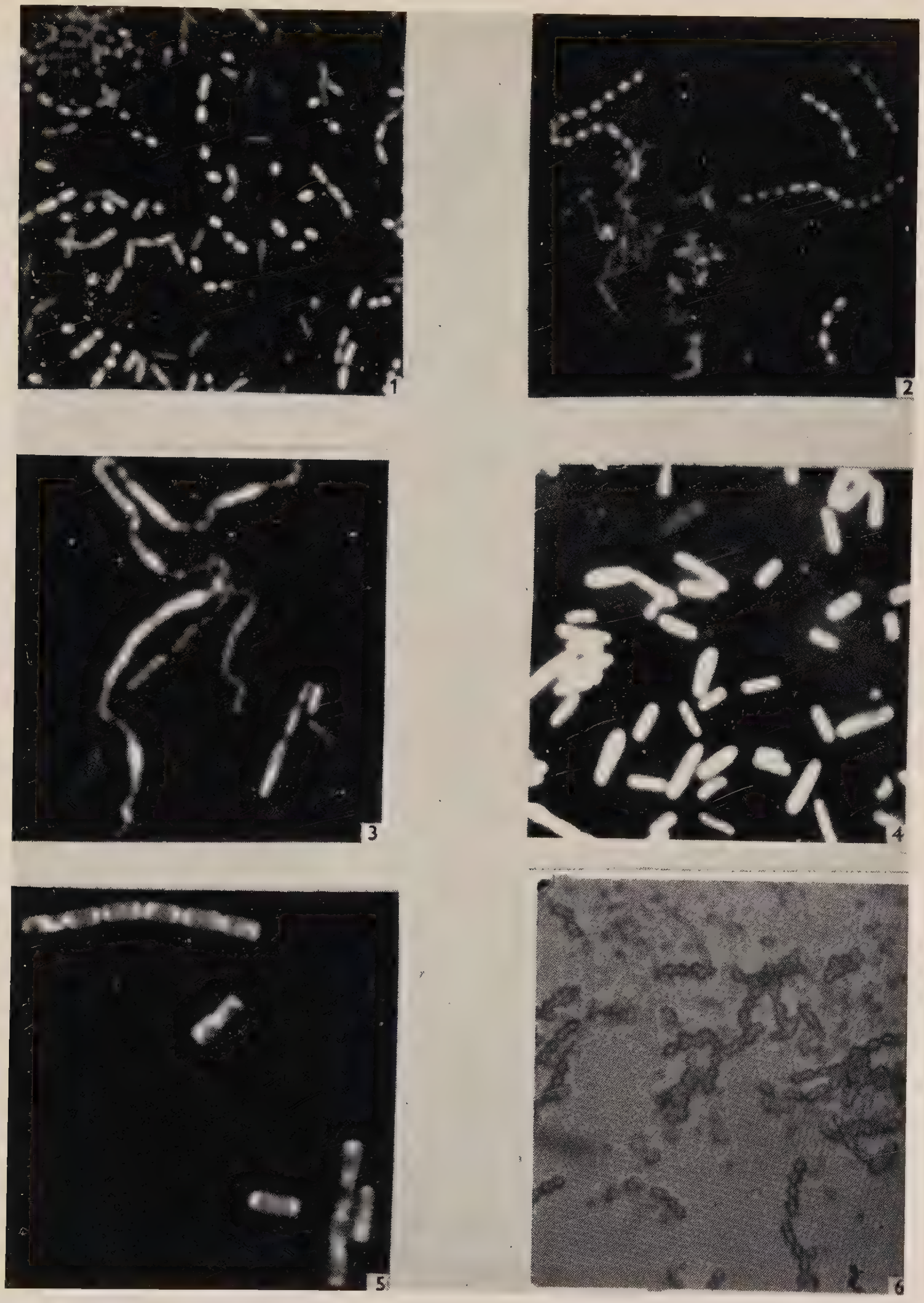

L. Bailey—Isolation of *Streptococcus pluton*. Plate 1

(*Facing p.* 48)

HUGHES, W. H. (1957). *J. gen. Microbiol.* 17, 49–58

A Reconsideration of the Swarming of *Proteus vulgaris*

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SUMMARY: Studies by cinemicrography confirm that the long forms of *Proteus vulgaris* (Hauser) seen during the swarming stage are morphologically indistinguishable from those induced by penicillin and other toxic agents. They appear only in cultures which are growing freely and when a threshold concentration of population has been reached; thereafter the organisms at the growing edge of the colony become more abnormal with each swarming. The normal small bacilli may begin to move before any long forms are present. In later swarmings, movement may be seen in small as well as in long organisms. It is suggested that the long forms are induced by a non-specific volatile agent which can not be detected when growth has been cleared from the medium. Its action is enhanced in cultures which are confined under a coverslip. Though it is partially neutralized by the action of catalase, conclusive proof of its nature is still lacking. When different strains are grown together long forms are soon produced. The agents causing this are specific and stable, producing their effect immediately, and are detectable in the medium after the primary culture has been cleared from it.

The swarming of *Proteus vulgaris* (Hauser) has received little attention recently. Since the monograph of Moltke (1927) few papers containing new observations have appeared. Mayr-Harting (Russ-Munzer, 1935) and Lominski & Lendrum (1947) offered different interpretations of the phenomenon. The interference of incompatible strains was studied by Dienes (1946, 1947) and by Cunliffe & Krickler (1953), who together with Kvittingen (1949, 1953) and Klieneberger-Nobel (1947), are among the few who have added anything to the earlier results.

During studies on the action of various damaging agents on bacteria (Hughes, 1956) it became clear that the spontaneous changes among swarming cells were like those caused by some chemical and the other traumata. Further, the mortality among these cells was exceedingly high, as Moltke (1927) had said. Since the earlier workers had reported their results, new techniques, notably timelapse cinemicrography and the use of the micromanipulator, have made the prolonged study of individual cells possible. It was decided to apply these techniques to study the nature of the phenomenon, and if possible to bring it into line with other examples of abnormal cell morphology.

METHODS

Technique 1. Buffered nutrient agar, 2–3 % was filtered through an F.C.B. Sterimet in a steamer to remove the debris and dead bacteria. These, though normally unimportant, may be mistaken for part of the implant of living cells when photographed. Before cooling the pH value was again adjusted to diminish precipitation of phosphates since these confuse the background. When required for use, the medium was melted and poured into heated Petri dishes, reasonably free from flaws, to give a uniform layer not more than 2 mm. thick. After cooling slowly at room temperature the medium was dried only long enough to remove surface moisture. The plates were inoculated with a straight wire as near the centre as possible.

At intervals sectors were cut, so that the whole of the growth from the site of inoculation to the colony edge, together with a clear area of unoccupied agar, was represented in the same relative amount as on the original plate. The extreme edge was removed with a scalpel, as its inclusion gives an optically unsatisfactory wedge and, when a coverslip is applied, the pressure is uneven and expressed fluid will collect. Both covered and uncovered preparations were examined at 37°. For prolonged observation the coverslips were sealed with paraffin wax. The plate from which the sector had been taken was also incubated as a control for any action due to the coverslip or to the separation of organisms from the colony.

Technique 2. While a coverslip resting on the agar was adequate for photography at normal speeds, it had disadvantages in timelapse work since the weight of the coverslip compressed the agar slowly and progressively and the field fell out of focus. This was overcome by making slide cells from dark gound slides. Two slats of glass 0·5 cm. wide were cut from a slide and cemented on to an intact slide 1·0 cm. apart. At the suggestion of Dr B. A. D. Stocker they were secured by Picein wax (W. Edwards and Son). Coverslips, 1·5 cm. wide, were also cut from slides. The organisms to be studied were dried on the coverslip, melted agar was poured between the two supports and the slip inverted on to this, pressed down and sealed with paraffin wax.

Technique 3. Where records of the whole area of the plate had to be made by timelapse the camera's own lens system was used.

Indirect oblique lighting from a 1000 W. studio lamp with a reflector was thrown by a mirror on to the Petri dish; this dish was supported on a sheet of glass and its optical defects rendered inconspicuous by running glycerol between the two surfaces in contact. A black velvet cloth was spread some distance below the glass. To prevent drying and to lift the cover of the dish out of the focal plane of the camera, a cylinder of Perspex of the same diameter as the dish and 8 in. high was seecurd to it by transparent tape. The upper end of the cylinder was closed with a sheet of plate glass and masked with a circle of black paper. To control condensation on this cover a bowl type ½ kW. electric fire was focused on it. Screens were suspended to protect the camera and other parts of the apparatus from direct light and heat.

Strains. The strains of *Proteus vulgaris* used in this work were 'P. 26', 'Utton' and 'Maye'. All had been repeatedly reselected with a micromanipulator and grown from single organisms. All these strains were of different types and on the same plate would not swarm into one another.

Examination of material and recording of results

The mounted sectors were examined by phase contrast, the microscope being enclosed in a Perspex incubator. The stages of growth were recorded by timelapse photography at a rate of one frame every 18 sec. When movement began, whether this was typical swarming, flagellar movement or the movement of normal cells, it was photographed at normal speed. Between exposures direct observation was maintained until further change was noticed, when this also was recorded. When the colony, by swarming, had covered a considerable area, a series of exposures were made from the edge to the site of the original inoculation.

After swarming the fate of the individual microbes was again followed by timelapse photography. Several different arrangements were made; (*a*) the preparation was left entire, covered with a slip and sealed; (*b*) it was left uncovered and followed until drying interfered with observation; (*c*) the swarming edge was cut from the rest of the colony and isolated; (*d*) swarm cells were transferred to fresh medium. Comparable sectors from the control plates were also examined.

Primary magnifications

Since the distance from the eyepiece to the camera gives a ×3 enlargement, to obtain a reasonable field it is necessary to decrease the magnification of the microscope. In general a ×20 objective with a ×6 or ×8 eyepiece was enough. The resolution was not high and for finer detail the ×95 oil-immersion lens with the Baker ×$1\frac{1}{2}$ eyepiece was more suitable.

Photographic details

After preliminary work with Kodak cinemicrofilm it was found that Kodak Super X reversal film was the most satisfactory as it allowed both timelapse photography, with a ×36 mercury green filter, and normal speed work, with a ×3 yellow-green filter.

Exposures of $\frac{1}{2}$ sec. were given since the shutter of most cinecameras give inconstant results on the first frame and this is smoothed over with the longer time exposure.

RESULTS

On solid medium growth begins normally and nothing distinguishes the Proteus cells from those of other bacillary organisms. This phase usually lasts for about 3 hr. at 37° and varies with the strain and the concentration of agar in the medium or the amount of drying.

When the colony has reached a diameter of about 8 mm. swarming begins. There will be a raised edge to the colony, and though no sign of swarming will be visible to the naked eye, continuous microscopic observation detects the first signs of activity. No long forms are then present but a small number of organisms become motile around the perimeter. This phase is transient and only prominent where islands of unoccupied medium have been left by the uneven spreading growth of the colony. Round these islands the movement is taken up by a complete line of organisms and continues until the space has been filled by them.

A further period of growth without visible independent movement of any of the organisms lasts for half an hour before the second phase of movement begins. The organisms separate out into two zones, the longer on the outside and the more motile, shorter ones between them and the main mass forming the old colony edge. The movement continues until the space has filled; these cells come in from the edge of the old colony, but during this time division is presumably also taking place. During the resting period which follows, small and large cells divide in the normal way without evidence that they are inhibited. The movements of small cells described above in covered preparations is also seen in uncovered ones.

The same sequence is repeated after another interval, but is now more obvious and there are three, rather than two, zones; movement can be detected under a hand-lens since there are now small irregularities projecting from the colony. Next to the solid colony is a palisade of long cells, then a space full of many active small cells and finally beyond this a further palisade. From both lines of long cells individuals swim out, their places being taken by others which have been swimming between the two palisades. After each of these cycles of activity, and as a result of the pattern left by the palisading, three rings are visible at the colony edge, easily misinterpreted as the result of three growth cycles instead of only one.

The fourth phase of activity is obvious to the naked eye and is the typical swarming reported by all previous workers. Groups of long forms push out over the medium, some are motile while others are inert and are simply carried along, being deposited at a distance from the colony edge and not infrequently picked up again by other passing groups. There is no evidence of negative chemotaxis since the groups return to the main mass when deflected, and at the peak of activity movement is random.

The maximum movement is not at the edge of the colony but some distance in. Here the violently active cells can be seen to divide while still motile and form trains of cells which follow each other through the lines of least resistance in the mass. At the site of the original implant only small forms are present; these show active movement though this is hampered by their numbers. Activity ends gradually, first in the small cells in the centre, then among the swarmers and last among the small forms derived from swarmers some distance from the edge. Subculture or continuous observation of long forms still undivided at the end of swarming shows that most of them are not viable, as was shown in the following experiments:

Experiment 1

The disk of agar carrying the growth up to the point where swarming began was cut from a colony, leaving a ring carrying mainly swarmers. Blocks were cut from this and mounted under coverslips and the fate of the swarmers recorded by timelapse photography.

Result. During the period up to 72 hr. no cell division was seen in covered preparations; the organisms became progressively less well defined and the contrast between them and the surrounding medium diminished.

Experiment 2

The organisms were transferred to fresh medium under a coverslip. They were examined visually at intervals of 30 min. by phase contrast and those giving rise to micro-colonies were counted.

Result. This experiment has been previously reported in detail (Hughes, 1953*b*). The number giving rise to normal small forms decreases after each successive swarming, being as high as 50 % with the first visible swarms, and falling to a fraction of 1 % in fourth and subsequent swarms.

The suppression of swarming by altering the concentration of the agar in the medium does not prevent the production of abnormal forms; although these strains are unable to progress on concentrated agar above 3 % the beating of their flagella can be detected under oil-immersion and, when the medium is moistened, they move freely on it. Drying of the medium during prolonged incubation is probably responsible for the irregularity of the later swarms mentioned by Russ-Münzer (1935). Sectoring may occur with the production of variants which are more tolerant of higher concentrations (Pl. 1, fig. 1). Unilateral drying demonstrates the effect of concentration particularly well (Pl. 1, fig. 2).

The nature of the toxic effect

The interaction of Proteus strains reported by Dienes (1946, 1947), and studied by Cunliffe & Krickler (1953), indicates there is some toxic effect when one strain acts on another. These workers showed that when two cultures of the same strain were planted on a plate together, when the swarms met they would join up and spread together. When different strains are on the same plate a line of separation is left between the swarms and the organisms on either side of the line are particularly bizarre in appearance. It has not previously been reported, however, that a similar action can be detected microscopically when strains are mixed and seeded together on solid medium, long forms then being produced after only a few divisions. This interaction cannot account for the development of swarmers from a pure strain since only compatible organisms are present. Again, it is well known that often when a broth culture is transferred to solid media some of the organisms may not divide and so become long forms. This is particularly well known with coliform organisms and with the salmonellas (Hughes, 1953*a*). These long organisms lyse early and are eliminated from the cultures, while the shorter bacilli establish themselves on artificial medium. This is entirely different from the Proteus long cell which only appears when colonial growth has reached an advanced stage.

While investigating this, most of Moltke's experiments were repeated with minor modifications and his results essentially confirmed. Only one or two need mention as of particular interest. He showed, for example, that when the agar on which a strain had grown was cleared of growth and remelted with added nutrients, or turned over and planted on the back, it was as acceptable to the strain as it was originally and no premature swarming occurred.

The experiment is simpler to perform when the organism is grown on a cellophan or collodion membrane; it is then unnecessary to remelt the agar, and clearing is more complete.

Experiment 3

A collodion membrane, average pore size 0·89 μ, diameter 4 cm. was placed in the middle of a nutrient agar plate. A drop of broth suspension was placed near the centre and the preparation incubated at 37°. Growth and swarming took place in the usual way.

The membrane with the growth was then removed leaving a ring of swarming cells beyond the edge. The centrifugal spread of the colony continued, but no swarming back on to the bare area took place.

This at first sight appears to be similar to the experiments of Lominski & Lendrum (1947), in which they showed that an area in the centre of a plate surrounded with growth is frequently not invaded but remains clear even when the whole of the rest of the plate has been occupied. This observation I have confirmed. It supports the idea of a negatively chemotaxic and, possibly, toxic agent, but it is important to note a difference between these authors' experiments and my own. The bare area in their cultures was essentially normal medium and was eventually occupied by a thin film of growth. The cleared one which had been under a membrane was depleted of nutrients and corresponded to that used by Moltke.

Experiment 4

The preparation was made as before but the membrane was removed with the entire culture before the swarms had reached the edge. The cleared plate was re-inoculated with a streak of organisms near to one side. Swarming across the plate was at first typical, but the site of the previous growth was avoided, leaving a bare area similar in shape.

Experiment 5

Blocks of agar were cut from this cleared area, mounted on slides and inoculated with cultures of the original strain P. 26, and incompatible strains 'Maye' and 'Utton'.

Result. Re-inoculation of the cleared area with a fresh inoculum of the same strain was followed by only a slow growth of cells of normal morphology. When, however, cells of an incompatible strain were used, long forms appeared as soon as if the strains had been grown in a mixture. Provisionally, then, we may assume that there are at least two toxic agents at work; one is retained in the medium, is relatively stable and the product of strains detected only in the presence of another, while a second is active only in crowded cultures and not specific in any way.

Peroxide accumulation would explain what we know of this latter agent. It might accumulate in areas of dense growth and probably disappear quickly from remelted agar or after clearing of the growth. It would not reappear unless growth again passed a certain threshold. Further, when a block of agar is cut from a plate with the swarmers on it and mounted under a coverslip, virtually all the cells will degenerate; but in a comparable area of the original growth, treated in the same way but uncovered, such extensive changes are not seen. That the action is in fact due to peroxide is not demonstrable. Proteus still swarms on catalase-rich medium, for example on blood-agar.

The action of catalase was shown in the following way:

Experiment 6

A plate of nutrient agar was divided into two halves by a ditch. One half was treated with a 1 % solution of catalase (containing 10 % of the active enzyme), the other was left as a control. At one end of the plate organisms were streaked at right angles to the ditch.

Result. The zones of swarming appeared sooner on the untreated side, being visible to the naked eye in 8 hr., while in the presence of catalase nearly 18 hr. elapsed. The width of the swarms was 0·8 cm. on the untreated side and 0·1–0·2 cm. on the treated.

When samples of swarmers were transferred to catalase agar, even under a coverslip, they recovered and divided normally as short cells, while the control cells under a coverslip lysed, or, if uncovered, remained abnormal. This is inconclusive since a similar but less marked result was obtained on any fresh medium.

Since it is held that under anaerobic conditions peroxide may not be formed, a further series of investigations were done on anaerobic cultures. If in a McIntosh and Fildes's jar plates were maintained in a humid atmosphere, the growth of Proteus rapidly spread without any zoning being seen and it was impossible to distinguish any effect on the cultures: dry anaerobic cultures were made in the following way:

Experiment 7

An anaerobic jar was prepared with a dish of phosphorus pentoxide in it and the jar evacuated an hour before hydrogen was introduced. Control plates were placed in identical jars but these were not evacuated. The jars were incubated either at room temperature or at 37°.

Result. The dry anaerobic plates showed the same zoning as the aerobic controls, and similar stages of spreading and of morphological change were reached in the same time. Finally the effect of the direct addition of hydrogen peroxide to the culture was tried.

Experiment 8

Peroxide was introduced into a slide cell in which *Proteus vulgaris* was growing on agar, in the hope that it would diffuse into the medium and that organisms near it would be obviously affected while those at a distance would remain normal.

Result. No changes in morphology were seen.

Experiment 9

Diffusion plates were prepared with ditches and punch holes in them. These were filled with peroxide. The plates were seeded with *Proteus vulgaris* and after various periods of incubation blocks were cut from them and the growth examined. The results were again negative. Finally one result was obtained suggesting that peroxide was itself playing some part.

Experiment 10

Into a row of 3 in. × ½ in. test tubes was placed 0·5 ml. broth. Serial dilutions of hydrogen peroxide at a nominal strength of 20 vol. were prepared in the usual way. To each tube was added one drop of an 18 hr. broth culture of *Proteus vulgaris*. A control without peroxide was also provided.

The preparations were incubated at 37° and examined after 1, 2, 4 and 17 hr. for growth and alteration of morphology. A second series was similarly prepared but the surface of the broth was covered with a layer of sterile liquid paraffin.

Result. After 2 and 4 hr. incubation the organisms in all concentrations above the equivalent of 0·6 vol. peroxide were non-motile. At 0·6 vol. long cells were seen. After 17 hr., growth was present up to 0·6 vol. and at this level only, organisms up to 7 times the normal maximum length for the strain were present. At no stage were any bulbs seen. Prolonged incubation did not bring about any further changes. The preparations sealed with paraffin were not more affected than the other series. The end-point for inhibition was also the same.

DISCUSSION

A number of apparently new observations justify this reconsideration of swarming. In the early stages no differences between Proteus and other bacillary forms could be seen. This is not in agreement with the views of Klieneberger-Nobel (1947) since she believed that there were differences between Proteus and coliform organisms at this early stage.

The early movement of small organisms need not be related to the formation of swarmers but it is not an artefact, due to the pressure of the coverslip on the raised edge squeezing out fluid from the medium, as Dr Mayr-Harting suggested (personal communication), and as I thought at first, since it is also regularly present in uncovered preparations. Further, the motility of the small organisms in the centre of the colony, difficult to study before phase contrast was discovered, suggests that there is a tendency to spontaneous movement by all the organisms and that this also may be independent of the formation of the swarmers though perhaps coincidental with it, both small and long forms being affected by the same stimulus to motility.

If other flagellated organisms, such as coliforms, are watched by time-lapses cinemicrography from the stage of single cells to a colony, it may be some time before any cells are seen to be motile and after the first cells have swum away a compact colony may still be left. There is a suggestion here that some time may pass before cells which are potentially motile reveal this characteristic on solid or semi-solid medium. The time at which this motility will be seen varies greatly from species to species, and even between strains of the same species and clones within the strain. It is, of course, well known that long forms are as easily produced in non-motile organisms as in motile ones. Thus motility and the production of the typical long forms hitherto regarded as swarm cells need not be related.

If it be true that there is some toxic agent responsible for the transformation of normal organisms into long forms then its effect should be greatest when there is maximum concentration, which would be in the middle of the mass of growth, yet the swarmers occur almost exclusively at the edge of the colony. Comparison with the penicillin-induced long forms offers an explanation. If the culture is seeded on a high concentration of penicillin + nutrient agar, long forms will develop immediately, while in lower concentrations some apparently normal divisions will take place first. On a medium which will not support growth no long forms are produced. Bigger showed (1944) that the resting cell was relatively resistant. In the same way in cultures of *Proteus vulgaris* the consumption of nutrients at the centre of the colony will encourage a resting stage there and the cells will neither develop abnormally nor degenerate. At the edge, although the concentration of toxic substances may be less, growth will be taking place and so both abnormal morphology and degenerative changes will be seen.

Just as the motility and the development of swarmers may be separate processes, so also there may be several toxic agencies at work. Interference between strains is not the same phenomenon as auto-intoxication. The inter-strain inhibitor remains in the medium after the original growth has been cleared from it, since re-inoculation with the same strain is followed by normal growth while with an heterologous strain growth is of long forms. One must postulate a group of strain-specific substances.

The general intoxication seen at the time of swarming is due to a more volatile agent. The two arguments for this are that (1) it cannot be detected in cleared medium, as Lominski & Lendrum (1947) and Moltke (1927) also found, and (2) it is far more potent in a culture under a coverslip than in one exposed to the air. This last observation harmonizes various statements on the viability of swarmers. Direct proof that the substance is hydrogen peroxide is not provided either by the addition of peroxide to the medium or by transfer to catalase medium. The former fails to produce the more advanced morphological changes although there are some long forms and the latter, while it delays the appearance both of long forms and of swarming does not inhibit them.

It is fair to conclude that there are several chemical agents concerned in the swarming of Proteus and although the nature of these is not known the long forms are induced in essentially the same way as by penicillin.

My thanks are due to my colleague, Dr Peter N. Cardew, head of the Photographic Department, for the supervision of all photographic techniques including the editing of the films.

Representative film sequences illustrating the results are available as a short sound film and may be obtained from the Photographic Department of St Mary's Hospital.

REFERENCES

BIGGER, J. W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilization. *Lancet.* ii, 497.

CUNLIFFE, A. C., & KRICKLER, M. S. (1953). A cultural test for the presumptive serological identity of *Proteus vulgaris. VIth Congr. int. Microbiol., Rome,* **1**, 400.

DIENES, L. (1946). Reproductive processes in Proteus cultures. *Proc. Soc. exp. Biol., N.Y.* **63**, 265.

DIENES, L. (1947). Further observations on the reproduction of bacilli from large bodies in Proteus cultures. *Proc. Soc. exp. Biol., N.Y.* **66**, 97.

HUGHES, W. H. (1953*a*). The origin of the L-form variants in anaerobic cultures of *Bacterium coli. J. gen. Microbiol.* **8**, 307.

HUGHES, W. H. (1953 *b*). Discussion in *Adaptation in micro-organisms. Symp. Soc. gen. Microbiol.* **3**, 250.

HUGHES, W. H. (1956). The structure and development of the induced long forms of bacteria in *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 341.

KLIENEBERGER-NOBEL, E. (1947). Morphological appearances of various stages in *B. proteus* and *coli. J. Hyg., Camb.* **45**, 410.

KVITTINGEN, J. (1949). Studies of the life-cycle of Proteus Hauser. *Acta path. microbiol. scand.* **26**, 24.

KVITTINGEN, J. (1953). Studies of the life-cycle of Proteus Hauser. *Acta path. microbiol. scand.* **32**, 170.

LOMINSKI, I. & LENDRUM, A. C. (1947). The mechanism of swarming of Proteus. *J. Path. Bact.* **59**, 688.

MOLTKE, O. (1927). *Contributions to the characterisation and systematic classification of* Bact. proteus vulgaris (*Hauser*). Copenhagen: Levin and Munksgaard.

RUSS-MÜNZER, A. (Mayr-Harting) (1935). Das Schwärmphänomen bei Bacillus Proteus. *Zbl. Bakt.* (1. *Orig.*), **133**, 214.

EXPLANATION OF PLATE

Fig. 1. Direct print of plate to show sectoring giving renewed growth on drying medium.

Fig. 2. Effect of unilateral drying of plate. Swarming only on medium where there is no concentration of agar.

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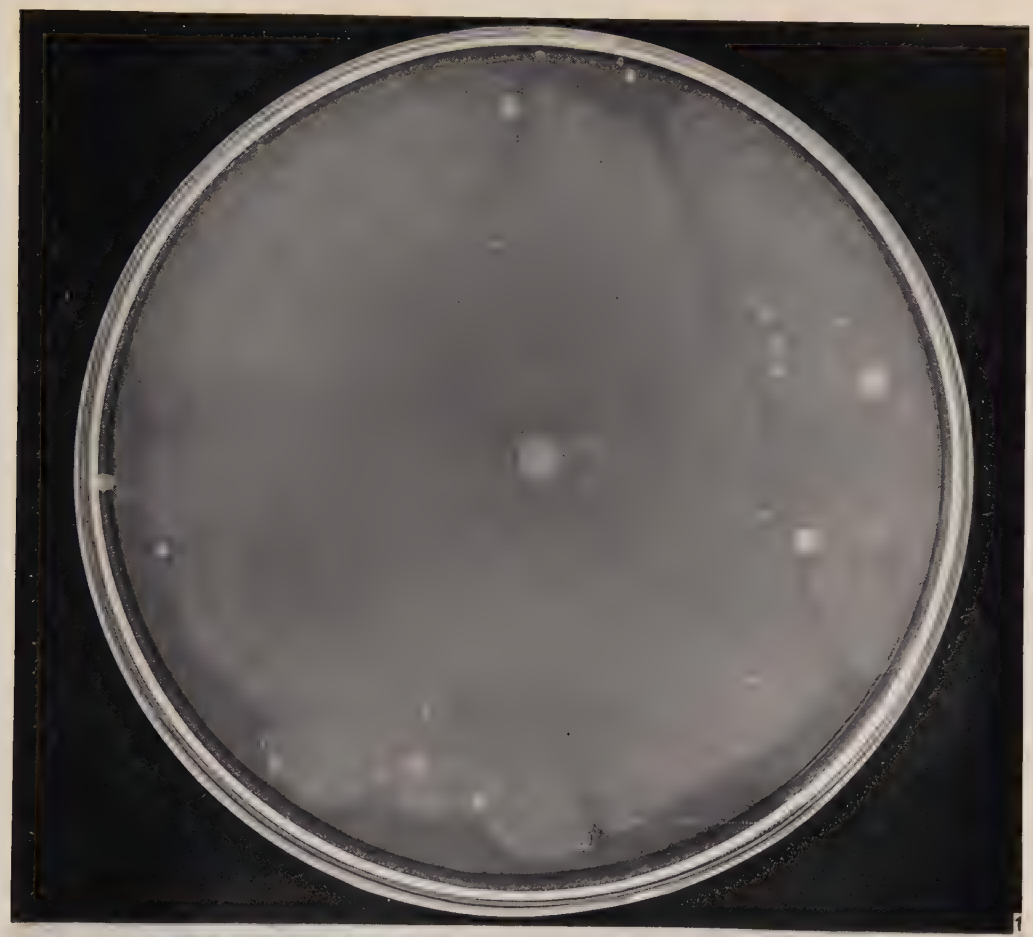

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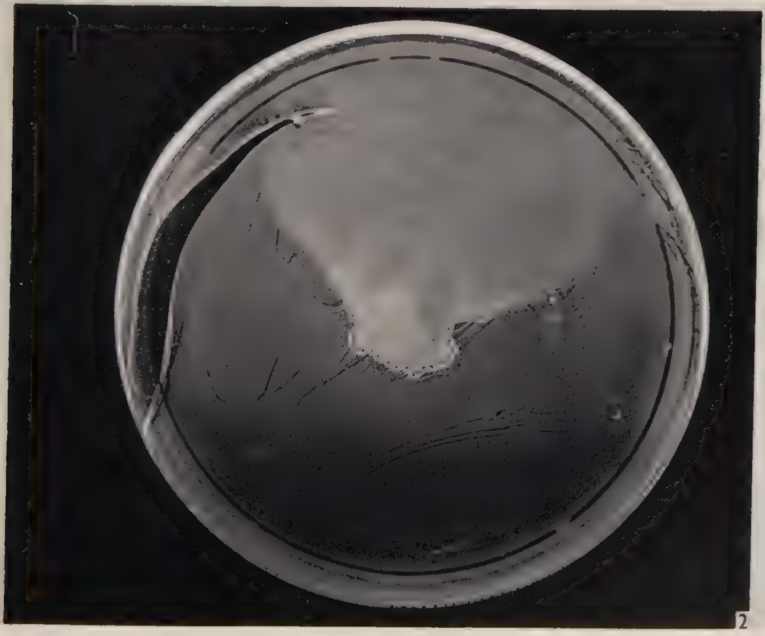

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W. H. Hughes—The swarming of Proteus. Plate 1

(*Facing p.* 58)

Jebb, W. H. H. & Tomlinson, A. H. (1957). *J. gen. Microbiol.* **17**, 59–63

The Minimal Amino Acid Requirements of *Haemophilus pertussis*

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SUMMARY: Of 13 sulphur-containing compounds tested only cystine, cysteine and glutathione satisfied the sulphur requirements of the strains of *Haemophilus pertussis* used when grown in a defined liquid medium containing 15 amino acids. Very small inocula (3–10 viable organisms) of 8 strains grew in a medium containing glutamic acid + cystine as the only amino acids added. Four strains were carried through 9 subcultures in this medium.

Previous work on the sulphur requirements of *Haemophilus* (*Bordetella*) *pertussis* has produced conflicting results. Different workers (Hornibrook, 1939; Verwey, Thiele, Sage & Schuchardt, 1949; Ungar, James, Muggleton, Pegler & Tomich, 1950; Proom, 1955) found that cystine, cysteine or glutathione supplied a requirement needed for full growth of large inocula. Imamura (1952), however, obtained growth with a wider variety of sulphur sources, including some inorganic compounds. The observations of Jebb & Tomlinson (1955), who used small inocula, suggested that cystine itself was not an essential requirement for growth. There are also conflicting results regarding the other amino acid requirements of *H. pertussis*. Imamura (1952) and Jebb & Tomlinson (1955) showed that *H. pertussis* would grow in media containing cystine + glutamic acid as the only amino acids. On the other hand, Proom (1955) stated that at least eight amino acids must be present in a medium if it were to support the growth of *H. pertussis* in serial subculture. A re-investigation of the sulphur and minimal amino acid requirements of *H. pertussis* has now been made.

METHODS

Strains. Some of the strains of *Haemophilus pertussis* used were isolated in this laboratory, and others were kindly supplied by Dr Elizabeth Rowatt (Lister Institute, Elstree). All strains used were freeze-dried from the first or second subculture after isolation; strain 724 was used in previous work (Jebb & Tomlinson, 1951, 1955).

Preparation of inocula. One- or two-day cultures on Bordet-Gengou medium were inoculated heavily on to this medium and incubated at 35° for 18 hr. The organisms were then washed off with saline, centrifuged, washed twice with saline, resuspended to known opacity and diluted for use. The standard inoculum was 0·1 ml. diluted suspension and was shown by colony counts to contain about 1×10^3 viable organisms.

Conditions of incubation and measurement of growth. Five ml. of medium were placed in 6 in. × $\frac{5}{8}$ in. test tubes, which had been bent at an angle of 45°, $1\frac{1}{2}$ in. from the open end. The tubes were incubated with the longer part horizontal, at 35°. The amount of growth was recorded as the mean optical density (extinction) of triplicate tubes, as described by Jebb & Tomlinson (1955).

Basal medium for the investigation of sulphur requirements. This medium was made up to four-fifths final volume so that 1·0 ml. volumes of the various supplements might be added aseptically to 4·0 ml. basal medium to give a final volume of 5 ml. The basal medium, when diluted to final volume, contained, (i) (amounts/l.): KH_2PO_4, 0·5 g.; NaCl, 6·2 g.; $MgCl_2$, 0·04 g.; $FeCl_3$, 0·01 g.; soluble starch, 1·5 g.; (ii) amino acids ($\times 10^{-4}$M): 2·7 L-alanine, 8·0 L-arginine, 6·0 L-aspartic acid, 100·0 L-glutamic acid, 3·0 glycine, 1·7 L-histidine, 4·0 L-isoleucine, 6·0 L-leucine, 5·0 L-lysine, 0·5 L-phenylalanine, 7·3 L-proline, 2·0 DL-serine, 2·0 DL-threonine, 4·0 L-tyrosine and 1·5 L-valine; (iii) 10^{-5}M-nicotinic acid; (iv) the following growth factors (μg./ml.): 6·0 guanine, 6·0 hypoxanthine, 6·0 xanthine, 1·0 haemin and 0·05 biotin. This basal medium was tubed in 4·0 ml. volumes and autoclaved at 10 lb./sq.in. (115°) for 20 min.

Medium G contained salts and starch at the concentrations given above and 0·01 M L-glutamic acid + 10^{-4}M L-cystine as the only amino acids added.

Medium GF consisted of medium G + the growth factors listed above.

The various growth factors and amino acids were obtained from British Drug Houses Ltd. and Roche Products Ltd. The glutamic acid was purified by recrystallization from water six times (see below).

RESULTS

Sulphur requirements

Because most of the amino acids used by Jebb & Tomlinson (1955) were natural L-isomers it was possible that cystine or cysteine was present as an impurity in one of them, and glutamic acid, being present in the highest concentration (0·01 M), was most suspect. When tested by the cyanide-nitroprusside reaction, the sample of L-glutamic acid which had been used was found to contain traces of cystine. A portion of this glutamic acid was, therefore, treated with hydrogen peroxide to oxidize the cystine (Rode, Lankford & Schuhardt, 1951) and another portion was recrystallized six times from water. In media made with these treated samples of glutamic acid no growth of *Haemophilus pertussis* appeared during 8 days of incubation, unless cystine or cysteine were added. In view of these results 50 g. of the L-glutamic acid specimen were purified by recrystallization and used for the preparation of the basal medium.

Solutions of the various sulphur compounds were made to contain equal concentrations of sulphur and the solutions of sodium sulphate, sulphite, thiosulphate and taurine were autoclaved to sterilize. The solutions of sodium hydrosulphite, sodium sulphide, cystine, cysteine, homocystine, methionine, glutathione, mercaptoacetic acid and cysteic acid were sterilized by filtration through a sintered-glass filter. After sterilization, dilutions were prepared to contain concentrations equivalent to 25·0 and 5·0 μg. S/ml. and 1·0 ml. volumes of these were added to tubes containing 4·0 ml. of basal medium. A series of tubes was seeded with the standard inoculum of each of four strains (including strain 724) and incubated for 7 days. No growth was observed with any sulphur compound except cystine, cysteine and glutathione; the growth with the latter compound was only one-eighth of that obtained with cystine at an equivalent concentration of sulphur.

As it was considered possible that other compounds might be utilized when autocalved in the medium, sodium sulphate, sulphite and thiosulphate, methionine, mercaptoacetate, homocystine and taurine were added to the basal medium before it was autoclaved. After autoclaving, the tubes were seeded with the standard inoculum of strain 724 and incubated for 7 days. No growth occurred.

To determine whether other compounds could be used in the presence of minimal concentrations of cystine, this compound was added to the basal medium in sufficient quantity (1·9 μg./ml., equivalent to 0·5 μg. S/ml.) to give one-sixth to one-eighth maximal growth and then sulphite, thiosulphate, methionine, mercaptoacetate or taurine (5·0 μg. S/ml.) was added. These compounds did not increase the growth of strain 724 above that which occurred in the medium containing a minimal concentration of cystine.

It appears, therefore, that of the compounds tested only cystine, cysteine and glutathione served as a source of sulphur for the strains of *Haemophilus pertussis* tested. The amount of growth of strain 724 with various concentrations of cystine or cysteine (added before and after autoclaving the medium) is shown in Fig. 1. The higher concentrations of cysteine, when added after the medium had been autoclaved, inhibited growth. When cysteine was autoclaved in the medium no inhibition occurred and a nitroprusside test showed that the cysteine had been converted to cystine. In this medium 38 μg. cystine/ml. (equivalent to 10 μg. S/ml.) were necessary for maximum growth. From these results it appears that our previous observation of slight growth in a medium to which cystine had not been added (Jebb & Tomlinson, 1955) could be accounted for by cystine present as impurity in the glutamic acid used. Our conclusion that methionine or sulphate might be used as a source of sulphur was not justified.

Amino acid requirements

The standard inoculum of *Haemophilus pertussis* strain 724 consistently grew in medium GF (about 200 μg. dry wt./ml. in 6 days). In order to be sure that glutamic acid and cystine were the only amino acids required it was necessary to establish that the observed growth was not due to the emergence of a less exacting variant and that growth could be maintained in serial subculture. Strain 724 was grown for 6 days in medium GF and the optical density of the culture measured; after dilution to give the standard size of inoculum a further lot of medium GF was inoculated; after 6 days of growth a second subculture was made. As all three cultures grew at the same rate it seems improbable that a variant was being selected.

In order to test more stringently the supposition that growth in medium GF was not due to a proportion of the organisms being less exacting than the majority, the smallest inoculum which would give growth was determined. Eight strains were grown on Bordet-Gengou medium and washed suspensions prepared; colony counts of these were performed, and dilutions made to provide a series of graded inocula. Each of three tubes of medium GF received 0·1 ml. of each dilution of inoculum and the tubes were incubated for 9 days. It would have been preferable to have used freshly isolated strains, but as these were not available the experiment was performed with cultures from freshly opened tubes of dried strains.

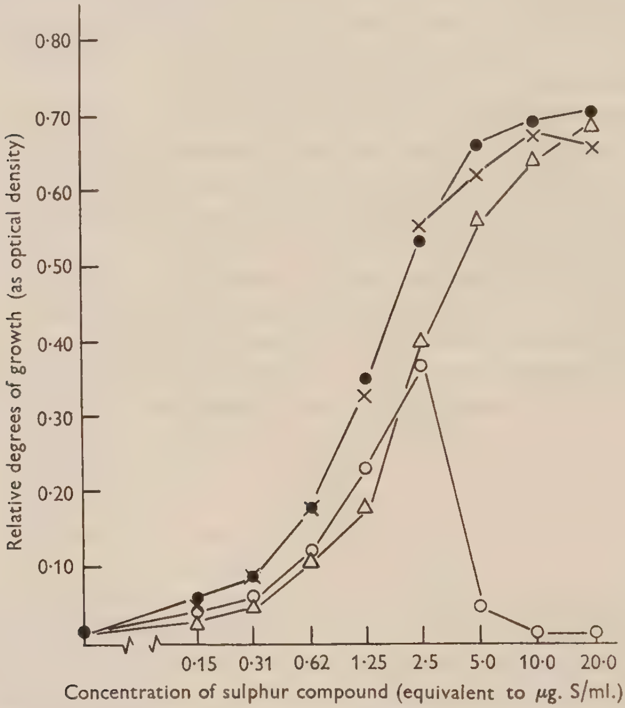

Fig. 1. Growth at 7 days, of *Haemophilus pertussis* strain 724, with various concentrations of cystine added before (×) and, after (●), autoclaving; cysteine added before (△), and after (○) autoclaving.

In every instance the inoculum from the $1/10^8$ dilution of the washed suspension grew and the colony counts showed that these inocula contained not more than 10 organisms which were viable on Bordet-Gengou medium. The high dilution eliminated the carry-over of significant amounts of amino acids with the inoculum and the occurrence of growth from an inoculum containing 3–10 viable organisms showed that either all the organisms were able to grow in the medium, or if a variant only were growing, that this variant must comprise 10–30 % of the whole population.

To confirm that growth could be maintained in serial subculture, the standard inocula of *Haemophilus pertussis* strains 724, 2439 and 10434 were put into medium GF, and after 6 days of growth a small loopful was transferred to fresh medium. Growth was maintained through 9 serial subcultures with no sign of diminution and it was concluded that the amino acid requirements of the strains of *H. pertussis* used were satisfied by glutamic acid + cystine.

Whereas nicotinic acid was the only vitamin added by Proom (1955) to his media, medium GF also contained guanine, hypoxanthine, xanthine, biotin and haemin, since these compounds had been found by Jebb & Tomlinson (1955) to stimulate growth. To determine whether the minimal amino acid requirements were more elaborate when nicotinic acid was the only growth factor present, medium G was supplemented with: (i) nicotinic acid; (ii) nicotinic acid + purines; (iii) nicotinic acid + purines + biotin + haemin; (iv) nicotinic acid + biotin + haemin. Strains 724, 8126, 2439 and 10434 were carried through four serial subcultures in these media. Although growth was slower in medium (i) than in medium (iii) there was no suggestion that it became poorer with successive passages, and there was thus no indication that the amino acid requirements were more complex in the absence of purines, biotin and haemin.

These findings with regard to amino acid requirements are at variance with those of Proom (1955), and the discrepancy may be due to the difference in time of incubation. Proom did not state how long he incubated his cultures but said that his methods were similar to those used by Knight & Proom (1950) who stated that 'growth was determined by visual observation...up to 3–4 days'; this might well be too short a period in which to observe the growth of *Haemophilus pertussis* in simple media, after the first few subcultures.

In work on the nutrition of a variable organism like *Haemophilus pertussis*, which is prone to degenerate in laboratory culture, it is preferable to use small washed inocula rather than to attempt serial passage of large inocula. When tested, however, by either method the strains of *H. pertussis* used grew in a medium containing only two added amino acids.

REFERENCES

Hornibrook, J. (1939). Cultivation of phase I *H. pertussis* in semi-synthetic liquid media. *Publ. Hlth Rep., Wash.* **54**, 1847.

Imamura, P. S. (1952). Growth requirements of *H. pertussis* with special reference to the production of thermolabile toxin. *Japan J. exp. Med.* **22**, 49.

Jebb, W. H. H. & Tomlinson, A. H. (1951). The catabolic activity of washed suspensions of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 951.

Jebb, W. H. H. & Tomlinson, A. H. (1955). The nutritional requirements of *Haemophilus pertussis*. *J. gen. Microbiol.* **13**, 1.

Knight, B. C. J. G. & Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.

Proom, H. (1955). The minimal nutritional requirements of organisms of the genus *Bordetella* López. *J. gen. Microbiol.* **12**, 63.

Rode, L. J., Lankford, C. E. & Schuhardt, V. T. (1951). Studies of sulfur metabolism of *Brucella suis*. *J. Bact.* **62**, 571.

Ungar, J., James, A. M., Muggleton, P. W., Pegler, H. F. & Tomich, E. G. (1950). The cultivation of *Haemophilus pertussis* in partially defined liquid media. *J. gen. Microbiol.* **4**, 345.

Verwey, W. F., Thiele, E. H., Sage, D. N. & Schuchardt, L. F. (1949). A simplified liquid culture medium for the growth of *Hemophilus pertussis*. *J. Bact.* **58**, 127.

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Pease, P. (1957). *J. gen. Microbiol.* **17**, 64–67

The Electron Microscopy of L-Forms Induced by Penicillin in *Proteus vulgaris*

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SUMMARY: When cultivated on membranes for electron microscopy, in the presence of 800 units penicillin/ml. and 10% (v/v) horse serum, a strain of *Proteus vulgaris* developed L-colonies in two stages. In the first cultures, the colonies consisted of naked protoplasts with a wide periphery of relatively disorganized protoplasm, and when further subcultured upon penicillin the entire colonies consisted of disorganized protoplasm. The typical bacillary form could be recovered by subculture of this material upon penicillin-free medium. In the process of regeneration the cultures passed initially through a stage in which they again consisted of protoplasts.

The mode of development of L-colonies has so far remained obscure. Klieneberger-Nobel (1949) suggested that the apparently structureless portion of the colony arises by coalescence of smaller units, whereas Dienes & Weinberger (1950–51) considered that the mechanism in question is one of fragmentation rather than aggregation. Numerous more recent studies have done little to elucidate this problem, which has been rendered more difficult by the very great fragility of the L-colonies. In a previous study (Pease, 1956*a*, *b*) this source of error was to some extent overcome by growing certain stages in *Spirillum* and *Vibrio* species, which strongly resembled L-forms, upon membranes for direct examination by the electron microscope. In the initial stages of this cycle the vibrios appeared to lose their customary curved regular outline and appeared in the form of irregular naked protoplasts, although each cell retained its single flagellum. From these protoplasts, small L-type colonies developed. Lederberg (1956) also noted the resemblance of the 'large bodies' to protoplasts.

Both the development from, and the reversion to the bacillary form, of more typical L-colonies induced in *Proteus vulgaris* under the influence of penicillin have now been observed by the same method, and it appears that both processes entail passage through a stage in which the bacteria grow in the form of naked protoplasts, without a firm cell wall.

METHODS

The strain of *Proteus vulgaris* used was a stock culture maintained in this laboratory. Cultures were made upon collodion membranes over nutrient agar plates containing 800 units penicillin/ml. and 10% (v/v) horse serum. The technique used was that of Hillier, Knaysi & Baker (1948; see also Pease, 1956*a*). Cultures were examined after *c.* 24 hr. of growth.

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RESULTS

When grown in the manner described, the entire culture consisted of typical L-colonies. The primary cultures upon penicillin-containing medium, when examined under the electron microscope, were seen to consist of groups of bodies of bacterial dimensions but oval or spherical in form and lacking the firmness of outline of normal bacteria. These bore a very strong resemblance to naked protoplasts. Surrounding these groups was a more diffuse zone of irregular material, consisting of larger and smaller granules (Pl. 1, figs. 1, 2).

When these primary L-cultures were further subcultured upon the medium containing penicillin and serum the appearance of the colonies under the electron microscope altered so that they appeared to consist entirely of the diffuse, irregular granular material (Pl. 1, fig. 3). All further subcultures on this medium retained this structure and form.

When subcultured on medium without added penicillin + serum, the early stages of the growth appeared to consist entirely of protoplast-like forms (Pl. 1, fig. 4). These protoplasts, which were in the process of regeneration towards the bacillary form, were firmer and fuller in appearance, and did not dry completely flat as did the degenerating states described already, but were in other respects similar. After a period ranging from 5 or 6 hr. to 24 hr. these protoplasts were transformed into vegetative bacilli. Further subcultures on medium without penicillin and serum consisted entirely of normal bacilli.

DISCUSSION

The phenomenon described in the foregoing section strongly resembles that which the apparently divergent hypotheses of Klieneberger-Nobel (1949) and of Dienes & Weinberger (1950–51) are intended to explain. It appears as if the first stage in the formation of the L-form from the normal vegetative organism is the loss of cell wall, leaving the protoplast. The second stage is a further disorganization of form and outline, presumably affecting the cell membrane, and resulting in a group of protoplasmic masses of irregular size and form. This is in accordance with the views of Dienes & Weinberger, and serves to explain the nature of the larger bodies, the breakdown of which they observed, although some of their findings may refer to the larger and smaller protoplasmic masses. The formation and development of L-forms in Proteus which were observed by phase-contrast in the living state by Stempen & Hutchinson (1951) bore a close resemblance to this suggested process. And the reversion from the L-form observed by the same technique by Medill & Hutchinson (1954) showed a group of 'large bodies', similar to protoplasts in form and dimensions, each one of which grew into a normal bacterium by a direct process which is entirely compatible with the concept that the cell wall was in process of regeneration. The resemblance between the 'large bodies' of the L-form and protoplasts has been commented upon by Lederberg (1956), and in relation to penicillin-induced lysis, by Hahn & Ciak (1957). This theory of the nature of L-forms is, however, at variance with that supported by certain workers, notably Tulasne (1953 and numerous other papers), that the L-forms represent a specialized dwarf reproductive cycle in bacteria. If the findings of Gale (1947) and Gale & Rodwell (1949) can be taken to indicate that the inhibitory action of penicillin is largely upon the cell envelopes of bacteria, then the loss or alteration of these structures may partially explain the relative resistance to penicillin of the L-form, especially since sensitivity appears to be restored or even enhanced immediately the bacillary form is resumed (Nermut, 1957). While this paper was in preparation, the same problem was discussed in an article by Park & Strominger (1957). Although dealing mainly with the effect of penicillin upon cell-wall synthesis, these authors offer the same explanation for the penicillin resistance of the L-form.

REFERENCES

DIENES, L. & WEINBERGER, H. J. (1950–51). The L-forms of bacteria. *Bact. Rev.* **15**, 245.

GALE, E. F. (1947). The assimilation of amino-acids by bacteria. 1. The passage of certain amino-acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.

GALE, E. F. & RODWELL, A. W. (1949). The assimilation of amino-acids by bacteria. 7. The nature of resistance to penicillin in *Staphylococcus aureus*. *J. gen. Microbiol.* **3**, 127.

HAHN, F. E. & CIAK, J. (1957). Penicillin-induced lysis of *Escherichia coli*. *Science*, **125**, 119.

HILLIER, J., KNAYSI, G. & BAKER, R. F. (1948). New preparation techniques for the electron microscopy of bacteria. *J. Bact.* **56**, 569.

KLIENEBERGER-NOBEL, E. (1949). Origin, development and significance of L-forms in bacterial cultures. *J. gen. Microbiol.* **3**, 434.

LEDERBERG, J. (1956). Bacterial protoplasts induced by penicillin. *Proc. nat. Acad. Sci., Wash.* **42**, 574.

MEDILL, M. A. & HUTCHINSON, W. G. (1954). The reversion of the L form of *Proteus mirabilis* into the rod form. *J. Bact.* **68**, 89.

NERMUT, M. V. (1957). L forms of bacteria and their role in the origin of penicillin resistance. *Nature, Lond.* **179**, 379.

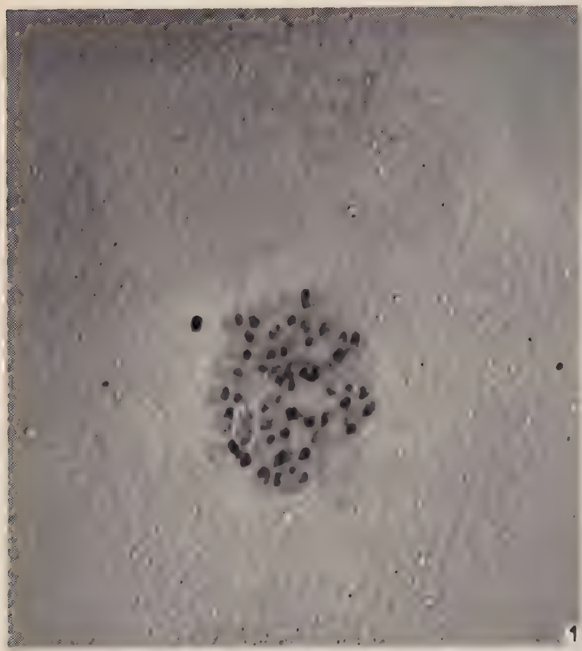
PARK, J. T. & STROMINGER, J. L. (1957). Mode of action of penicillin. *Science*, **125**, 99.

PEASE, P. (1956*a*). The gonidial stages in *Spirillum* spp. and *Vibrio* spp. *J. gen. Microbiol.* **14**, 672.

PEASE, P. (1956*b*). Electron microscopy of L-forms induced in *Vibrio* spp. by the action of penicillin. *J. gen. Microbiol.* **15**, xiv.

STEMPEN, H. & HUTCHINSON, W. G. (1951). The formation and development of large bodies in *Proteus vulgaris* OX-19. 1. Bright phase contrast observations of living bacteria. *J. Bact.* **61**, 321.

TULASNE, R. (1953). Le cycle L et les formes naines des bactéries. Symposium: *Citologia Batterica*. VIth Congr. int. Microbiol., Rome.



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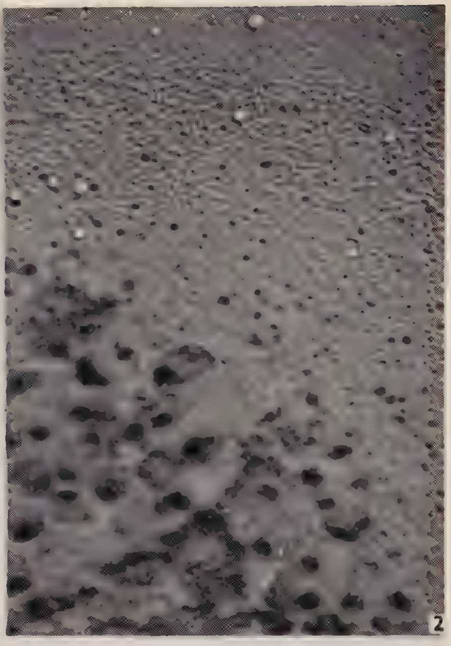

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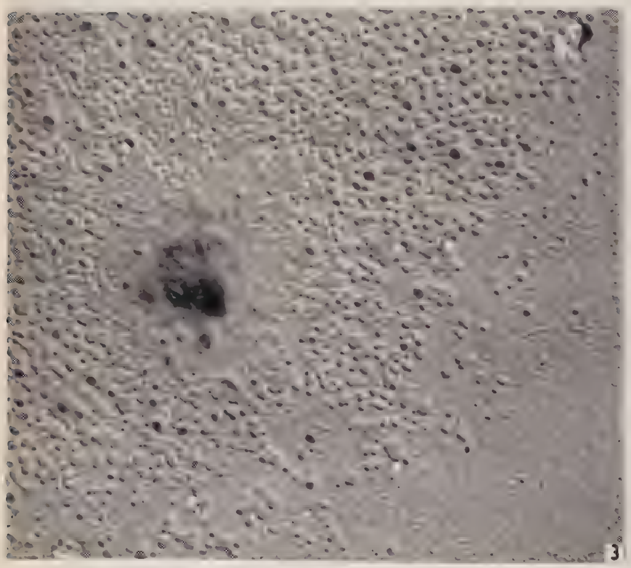

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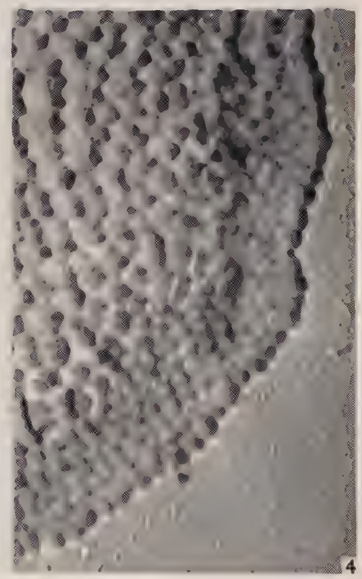

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P. PEASE—L-FORMS OF PROTEUS. PLATE 1

(*Facing p.* 67)

EXPLANATION OF PLATE

Fig. 1. *Proteus vulgaris* L-colony, first subculture (24 hr.) on penicillin serum medium. Showing central group of degenerating protoplasts surrounded by diffuse matter. Electron micrograph, gold-palladium shadowed. × 5,000.

Fig. 2. As fig. 1, sector of colony. × 10,000.

Fig. 3. Second subculture (24 hr.) on penicillin + serum medium. Diffuse matter mainly, with variously sized granules. Electron micrograph gold-palladium shadowed. × 15,000.

Fig. 4. First subculture of L-forms upon medium without added penicillin and serum (18 hr.). The colony consists entirely of protoplasts. These are fuller in appearance than those in the degenerating stages. Electron micrograph gold-palladium shadowed. × 4,000.

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Beale, G. H. & Kacser, H. (1957). *J. gen. Microbiol.* 17, 68–74

Studies on the Antigens of *Paramecium aurelia* with the Aid of Fluorescent Antibodies

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SUMMARY: Preparations of *Paramecium aurelia*, which had been immobilized by placing the organisms in a solution containing antiserum conjugated with fluorescein (conjugated antiserum), were examined by fluorescence microscopy. Unfixed paramecia accumulated fluorescent antibody in a thin layer around the entire surface of the organisms, and in globules at the clumped tips of the cilia, but not in the cilia themselves. No fluorescence was seen in the nuclei or the cytoplasm, but the food vacuoles became brightly fluorescent when the paramecia remained in conjugated antiserum for a few hours. Paramecia, which had been fixed with osmic acid and subsequently treated with fluorescent antibody, showed a faint fluorescence along the whole lengths of the cilia. When transformation from one serotype to another took place, the change in ability to take up a given kind of fluorescent antibody was seen to occur uniformly over the whole surface of the organism. It is concluded that the immobilization antigen is a fluid substance which covers the whole surface of the paramecia and is exuded into the medium under certain conditions, especially when homologous antibody is present.

Extensive studies have been made on the genetics of antigen variation in *Paramecium aurelia* by Sonneborn, Beale and others (for references, see Beale, 1954, 1957). In all such work only one immunological reaction has been used, namely, the immobilization of the organisms which takes place when living paramecia are placed in a fluid containing dilute homologous antiserum. It is known that this immobilization reaction is associated with a clumping of the cilia, and for this reason the antigens concerned have been generally referred to as the 'ciliary antigens'. However, definite evidence that these antigens are derived from part of the ciliary structure has never been brought forward.

The paramecium antigen system, with its complex array of determining factors in the nucleus, cytoplasm and environment, has been considered as a model, valuable for the understanding of mechanisms of cell differentiation generally (Sonneborn, 1947; Beale, 1952). It is therefore important to obtain as much information as possible concerning the nature and location of the final products in the system, namely the specific antigens themselves. The elegant technique developed by Coons and collaborators (Coons, Creech, Jones & Berliner, 1942; Coons & Kaplan, 1950), offers a means for localizing the sites of deposition of antibodies in tissues, and thus enables inferences to be drawn regarding the sites of the corresponding antigens. Essentially the method involves conjugating the antibodies in suitable antisera with a fluorochrome such as fluorescein, applying the conjugated antiserum thus obtained to the tissue under investigation, washing away the fluorescent solution, and observing the treated material by fluorescence microscopy, that is, under ultraviolet (u.v.) irradiation. The characteristic bright green fluorescence of fluorescein indicates the site of deposition of conjugated antibody. In such tests it is important to set up suitable controls, since the conjugated antiserum contains other conjugated proteins which may be taken up non-specifically by the material under study and give spurious indications of an uptake of fluorescent antibody. Furthermore, exchange of fluorescein from antibody to other material must be eliminated as a possible cause of localized fluorescence. With the antigens of *Paramecium*, such controls may be conveniently obtained by exposing paramecia of a different serotype to precisely the same treatment as the homologous organisms, as will be described.

In the experiments here reported, whole paramecia were exposed to fluorescent antiserum. Hence, only the external parts of the cell were studied. In future work it is hoped to apply fluorescent antiserum to sectioned material, and to examine whether there are substances related to the immobilization antigens in the interior of the organism, in the cytoplasm and nucleus, where it is known from genetic evidence that the determinants of the immobilization antigens are situated.

METHODS

A sample of immune rabbit antiserum, developed against *Paramecium aurelia* stock 168 (variety 1), serotype G, was used. Paramecia of serotype 168G were the homologous test objects; and types 168D and 90G were the non-homologous control organisms. Details concerning the preparation of antisera have been described previously (Sonneborn, 1950; Beale, 1954, 1957). Suspensions of broken-up paramecia were used for injections into rabbits. The conjugation of the antiserum with fluorescein was affected by the method of Coons & Kaplan (1950). Whole serum was used. We are greatly indebted to Dr A. B. Roy for carrying out this part of the work, and for supplying a sample of fluorescein isocyanate.

The conjugated antiserum obtained was diluted about one part in twenty-five with distilled water, and this was the solution used in all tests. Samples were kept frozen, and thawed as required. The comparatively high concentration of conjugated antiserum which had to be used was a consequence of the considerable lowering in titre of the antiserum, as measured by the immobilization reaction (from 1/800 to 1/100) due, presumably, to the chemical procedures used in conjugating antibody with fluorescein.

Three methods of preparing the paramecia for examination by fluorescence microscopy were used: (1) live paramecia were simply treated with dilute fluorescent antiserum, washed with dilute buffered Ringer solution, and examined while still living, though immobilized; (2) after treatment and washing as above, paramecia were fixed by exposing them to osmic acid vapour for 30 sec. (or by placing them in very dilute buffered osmic acid solution); (3) paramecia were first fixed in osmic acid, then placed in the dilute fluorescent antiserum, and finally washed and examined.

In order to obtain satisfactorily bright fluorescent preparations (i.e. suitable for colour photography), it was found necessary to expose the paramecia very thoroughly to the conjugated antiserum, e.g. living paramecia were treated about three times longer than necessary to bring about immobilization, and osmic acid-fixed specimens were left in the fluorescent antiserum (at a dilution of about 1/50) overnight.

After treatment, the paramecia were placed on a clean glass slide in a small drop of dilute (1/10) Ringer solution, and a u.v.-absorbing coverslip placed on top, supported at the sides by two thin pieces of coverslip. A 250 W. mercury vapour lamp was used as a source of u.v. radiation, and visible light was excluded by suitable filters. Glass lenses were found to be adequate. Most of the preparations were examined with a dark ground condenser.

RESULTS

Before describing the effect of fluorescent antiserum on paramecia, we may consider briefly the immobilization process in so far as it can be studied with non-fluorescent material observed under the phase-contrast microscope. As shown in Pl. 1, figs. 1 and 2, when paramecia were immobilized by antiserum, the cilia clumped together at their tips, where there was an accumulation of lumps of 'sticky' substance. Such material may also accumulate in the solution around the paramecia, especially at the posterior ends of the ciliates. Similar observations have been recorded by other workers (e.g. Harrison, 1955), but there has been no proof that these accumulations of 'sticky' material actually contained antigen-antibody complexes. It is possible that bacteria and other debris from the medium contributed a large part of the bulk of the lumps.

The cytoplasm of paramecia which had been immobilized by specific antiserum continued to circulate actively; and even the cilia could be seen to be in a state of agitation, making such irregular contractions and lateral movements as are possible when the tips are stuck together. The special gullet cilia, moreover, were apparently quite unaffected by specific antiserum. Trichocysts could occasionally be seen to be extruded as a consequence of treatment with antiserum, and this was especially pronounced in immobilized paramecia which were subsequently fixed with osmic acid vapour.

When fluorescent antiserum was applied to paramecia of homologous serotype (i.e. type 168G) by method (1) (see above, p. 69), and examined under u.v. radiation, fluorescence appeared in a thin layer around the entire surface of the organism, in globules (waving back and forth) at positions corresponding to the clumped tips of the cilia, and in granules interspersed in the 'sticky' material at the posterior ends of the paramecia. The cilia themselves were not fluorescent, except for the large stationary caudal cilia, which were sometimes faintly green along their length. There was no fluorescence in the gullet. Finally, a few food vacuoles were brightly fluorescent, and could be seen circulating around the cytoplasm. The nuclei and cytoplasm were invisible when examined by fluorescence microscopy.

Non-homologous paramecia of serotypes 90G or 168D (i.e. those differing from type 168G by either genic or cytoplasmic factors, respectively) after being placed in the conjugated anti-168G serum, did not fluoresce on the surface at all, and were of course not immobilized. Such paramecia did, however, ingest the fluorescent material, and when allowed to do so for 12 hr. or more showed a large number of intensely green fluorescent food vacuoles. It is assumed that the accumulation of fluorescent material in the food vacuoles had nothing to do with specific uptake of antibody by antigen, but was purely mechanical.

The specificity of the uptake of the fluorescent antibody by paramecia could be very clearly demonstrated with the aid of pairs of conjugating animals. In each pair, it was so arranged that one animal was of serotype 168G, while the mate was of some other serotype. It was found to be important to have the pairs firmly joined together before treating them with fluorescent antiserum, and this was achieved by allowing conjugation to go on normally for 2 hr. or more at 27° in ordinary culture fluid. Such pairs exhibited a remarkable differential uptake of fluorescent material: the homologous member (168G) showed a green deposit around the surface, the other did not show the slightest trace of fluorescence on the surface, even in those regions where the cilia of the two mates were extremely close together. Both members of the pair, but particularly the non-homologous one, sometimes accumulated fluorescent material in some of the food vacuoles.

It might be argued that paramecia which have been treated with homologous antiserum and are therefore in a 'sticky' condition, would be liable to take up non-specifically any material present in the medium, and this would include, in these experiments, fluorescent substances. To check this point, the experiment described in the previous paragraph was also performed in a modified form, as follows. Pairs of conjugating paramecia (of diverse serotype) were treated first with a mixture of ordinary, non-fluorescent antisera capable of immobilizing both members of a given pair, and subsequently with fluorescent antibody effective against only one of them. Here also it was found that only one member of each pair became fluorescent, even though the other member had been immobilized, and was presumably in a 'sticky' condition.

All observations made on living paramecia, prepared according to method (1) had to be made quickly, since the intense beam of u.v. radiation kills the organism in a few seconds. Amongst effects observed during this killing by u.v.-irradiation was a movement of green fluorescent material away from the paramecium into the solution.

After preparation by method (2), i.e. with final fixation in osmic acid, the paramecia showed essentially the same distribution of fluorescence as in the living material described above, but the differentiation between fluorescent and non-fluorescent regions was not quite as sharp, partly due to the fact that in fixed material the protoplasm itself showed a faint bluish fluorescence. Fixed material had the advantage, however, of remaining unchanged after exposure to u.v. radiation for some time, though eventually the green colour was bleached out. As mentioned above, trichocysts were liable to be thrown out in quantity when paramecia were immobilized by antiserum and fixed with osmic acid. When this happened, the mass of extruded trichocysts brought out with it quantities of green fluorescent granules similar to those which collect at the tips of the cilia. The extruded trichocysts themselves were sometimes fluorescent, sometimes not. It is possible that trichocysts which are extruded during the period of exposure to conjugated antiserum become coated with fluorescent material, while those which are brought out by the final fixation are non-fluorescent.

Paramecia which were prepared by method (3), i.e. fixed with osmic acid before being treated with conjugated antiserum, showed an appearance somewhat different from that described above. The cilia of organisms treated according to method (3) did not clump at their tips, and there were no fluorescent granules either at the tips of the cilia or adhering to the exterior of the organism. What was very distinctive here was that the cilia were clearly fluorescent along their entire lengths. The pellicle was, as before, fluorescent around the whole surface. Finally, there were of course no fluorescent food vacuoles since the paramecia were dead before being put into the diluted conjugated antiserum.

It should be emphasized that the uptake of fluorescent antibody by cilia which had been previously fixed with osmic acid was highly specific, as shown by the failure of antigenically non-homologous paramecia, treated in precisely the same way, to show any such uptake.

After finding that fixation with osmic acid did not destroy the ability of paramecia to take up homologous antibody specifically, a number of other treatments were tried, such as fixation with formaldehyde and heating to 70° for 2–3 min. Such treatments resulted in the conversion of the paramecia to a form in which all of them, whether antigenically homologous or not, acquired a fluorescent coating. It is presumed that this non-specific uptake had nothing to do with absorption of specific antibodies by antigens on the surface of the paramecia, for it should be remembered that the diluted fluorescent antiserum contained other proteins conjugated with fluorescein, in addition to the immobilizing antibodies.

Observations were made on the distribution of antigen in paramecia which were in the process of transforming from one serotype to another. The process by which paramecia transform, under the influence of various environmental treatments, has been described elsewhere in detail (Beale, 1954, 1957). Paramecia of serotype 168G can be very easily caused to give rise to organisms of serotype 168D by growth at a high temperature (27°–35°). In the present studies, some organisms of type 168G were separately isolated into culture fluid and incubated at 35° for various periods up to 12 hr., by which time the transformation to 168D was practically complete. At intervals during the transformation process samples were taken, fixed with osmic acid, treated with fluorescent antiserum, washed and examined. It was found that as the reaction of the organisms to anti-168G serum diminished, so did their ability to take up specific fluorescent antibody. The diminution in ability to take up the specific fluorescent antibody was seen to be a gradual process, though it was not possible to decide whether the cilia, or certain regions of them, lost the ability to take up the fluorescent antibody at an earlier stage than the pellicle, because the fluorescence of the cilia was at all times faint. What, however, was clear was that the change in the antigen occurred uniformly all around the organism and did not occur only at regions where new cilia and pellicle were being developed. In this connexion it is worth recalling that during fission there is thought to be an abundant production of new cilia in a band approximately around the equator of the animal (Wichterman, 1953), and fission occurred during transformation.

CONCLUSIONS

From the observations reported in this paper, it is concluded that the antigen concerned with the immobilization reaction in paramecia is found on the pellicle and on the cilia, but may diffuse into the medium under the influence of certain treatments. Fixation with osmic acid does not destroy the antigen, but prevents it from diffusing away from the site at which it is formed, possibly by making the external membrane impermeable to the antigen, though not, apparently, to antibody. From the fact that during transformation, in actively growing organisms, from one serotype to another, there is a uniform change in the antigen all over the surface, it is deduced that the antigen is not associated with any particular structure, such as the cilia. Rather would it seem that the antigen is a fluid substance which can cover any part of the exterior surface of the paramecium. When homologous antibody is added, it is thought that there is an exudation of antigen, which unites with the antibody and forms the 'sticky' lumps at the tips of the cilia and elsewhere. The precise cause for this accumulation is not clear, but it may be mechanical. The antigen-antibody product never sticks to the cilia of non-homologous animals, however close they may be.

Views somewhat similar to the above were put forward by Harrison (1955), arising out of observations, with phase-contrast microscopy, on the immobilization reaction in Paramecium, Tetrahymena and Colpoda. This author considered that the 'slimy accumulation' formed around ciliates when homologous antibody is added, is an antigen-antibody product, since it appeared in any and all combinations of strains of ciliates and antisera which gave precipitative and complement-fixation reactions, but in no other situations. Harrison also noted a loss in volume of paramecia during immobilization, and assumed this to be due to the exudation of the antigen. Such a pouring out of exudate, which passes out into the surrounding medium and combines with antiserum, was described by Robertson (1939), with Tetrahymena. Amongst other observations recorded by her was one to the effect that the cilia were not attacked by the antiserum, but were withdrawn from the antigen-antibody 'sheath', and could later resume normal activity.

In view of the diffusible properties of the paramecium antigen it seems likely that the latter is constantly being exuded into the water in which the paramecia are swimming, but we have no definite proof that this is so. It is, however, known that a soluble antigen can be readily extracted from the paramecia (Finger, 1957; Van Wagtendonk, van Tijn, Litman, Reisner &

Young, 1956) and that this soluble antigen specifically lowers the titre of the antibodies concerned with the immobilization reaction.

As a result of the conclusions arrived at in this paper, we feel that the term 'ciliary antigen' is inappropriate for a substance which is found, not only on (or in) the cilia, but also, and most abundantly, on the pellicle, and which may well be continuously passing into the medium. 'Immobilization antigen' would in our opinion be a better term.

We wish to thank Drs A. B. Roy and R. Alexander for much help in the preparation of the fluorescent antiserum and for technical advice generally.

REFERENCES

Beale, G. H. (1952). Antigen variation in *Paramecium aurelia*, variety 1. *Genetics*, **37**, 62.

Beale, G. H. (1954). *The Genetics of* Paramecium aurelia. Cambridge University Press.

Beale, G. H. (1957). The antigen system of *Paramecium aurelia*. *Int. Rev. Cytol.* **6**, 1.

Coons, A. H., Creech, H. J., Jones, R. N. & Berliner, E. (1942). The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immunol.* **45**, 159.

Coons, A. H. & Kaplan, M. H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.* **91**, 1.

Finger, I. (1957). Immunological studies of the immobilization antigens of *Paramecium aurelia* variety 2. *J. gen. Microbiol.* **16**, 350.

Harrison, J. A. (1955). General aspects of immunological reactions. Chap. 7 in *Biological Specificity and Growth* (ed. E. G. Butler). Princeton University Press.

Robertson, M. (1939). A study of the reactions *in vitro* of certain ciliates belonging to the *Glaucoma-Colpidium* group to antibodies in the sera of rabbits immunized therewith. *J. Path. Bact.* **48**, 305.

Sonneborn, T. M. (1947). Developmental mechanisms in Paramecium. *Growth* **11**, 291.

Sonneborn, T. M. (1950). Methods in the general biology and genetics of *Paramecium aurelia*. *J. exp. Zool.* **113**, 87.

Wagtendonk, W. J. Van, Tijn, B. Van, Litman, R., Reisner, A. & Young, M. L. (1956). The surface antigens of *Paramecium aurelia*. *J. gen. Microbiol.* **15**, 617.

Wichterman, R. (1953). *The Biology of Paramecium*. New York: The Blakiston Co.

EXPLANATION OF PLATE

Fig. 1. Unimmobilized paramecium, fixed in osmic acid vapour, photographed in phase-contrast microscope. × 500.

Fig. 2. Paramecium immobilized with homologous antiserum, then fixed and photographed as Fig. 1. × 500.

(*Received* 16 *January* 1957)

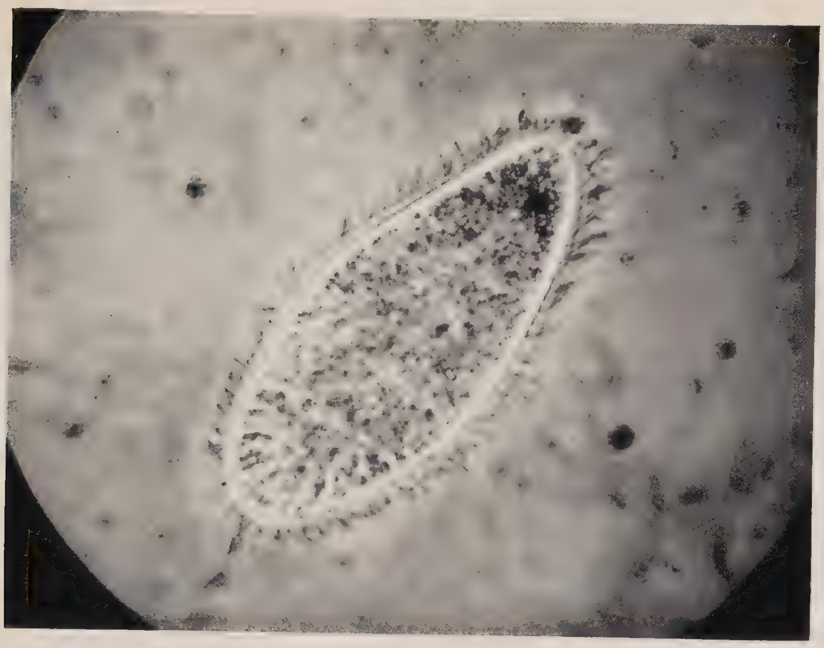

Fig. 1

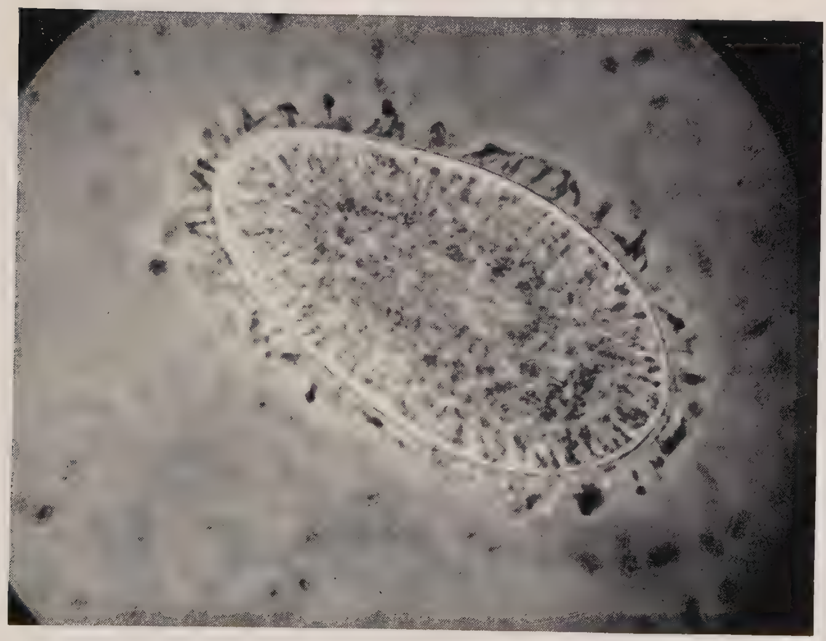

Fig. 2

G. H. Beale and H. Kacser—Antigens of *Paramecium aurelia*. Plate 1

(Facing p. 74)

BRADLEY, D. E. & WILLIAMS, D. J. (1957). *J. gen. Microbiol.* 17, 75–79

An Electron Microscope Study of the Spores of some Species of the Genus *Bacillus* using Carbon Replicas

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SUMMARY: A method for the preparation of carbon replicas of Bacillus spores is described. Micrographs of these replicas reveal much more of the surface detail than direct electron micrographs. Rib formations were found on five different species and the micrographs obtained suggest that the sculpturing varies according to the species.

Hitherto, little work has been carried out on the study of spore surfaces, partly because of lack of suitable techniques, and partly because the likelihood of any surface structure being present seemed remote. The electron microscope provides the necessary resolution required to reveal submicro-structure, and spores can be examined by thin sectioning, by replica techniques, or by direct examination. Thin sections of *Bacillus megaterium* and some other spores (Robinow, 1953; Van den Hoof & Aninga, 1956) show what may be seen by this method. The internal structure of the spore is clearly shown, but there is no indication of any surface detail, and the technique seems unsuitable. Because of the high density of the spores to electrons, direct examination (Knaysi, Baker & Hillier, 1947; and see Pl. 4, fig. 11) provides only an outline of the spore and little information about the nature of the surface. The most obvious approach is the use of a replica method, but few of the existing techniques can be used for the examination of biological materials. Nevertheless, a variation of the carbon replica technique (Bradley, 1954) was successfully used in a study of yeast bud scars (Bradley, 1956), and a modification of this method has provided a simple means of studying spore surfaces. The results are both striking and unexpected. Instead of the smooth surface which was expected, deep sculpturing was found. Moreover, this sculpturing differs widely from species to species.

METHODS

Preparation of spore suspension

A medium of the following composition was used: Peptone (Oxoid), 6 g.; Bacto tryptone, 3 g.; Yeastrel, 3 g.; Lab. Lemco (Oxoid), 1·5 g.; agar, 25 g.; Mn^{++}, 1 p./m. (as sulphate or chloride) in 1 l. distilled water; pH 7·0; sterilized at 121° for 20 min.

Nutrient agar slope cultures of the organism (24 hr. at 37°) were emulsified with sterile distilled water and approximately 1 ml. inoculated into Roux bottles (1 l.) containing 140 ml. of the above medium. After incubation for 1 week at 37°, spores were harvested in distilled water and shaken for 1 hr. in 4 oz. screw-cap bottles containing glass beads. Suspensions were washed by centrifuging four times with distilled water, heated in a water bath at 80° for 20 min. to kill vegetative organisms, and finally stored in a refrigerator (*c.* 5°) until required. In some cases this method of preparation did not provide suspensions free from vegetative organisms. This was particularly so with *Bacillus sphaericus.*

Organisms used

Bacillus subtilis 4, raw tanker milk;* 18, NCIB 8057; 41, Knight & Proom (1950), CN 2745; 22, raw tanker milk.*

B. licheniformis 92, NIRD stock culture; 93, NCIB 6816; 94, NCIB 7224.

B. circulans bulk tanker milk.*

B. polymyxa bulk tanker milk.*

B. brevis bulk tanker milk.*

B. sphaericus from J. Wolf, University of Leeds.

The replica technique

The replica technique is very simple. The spores are coated with a thin film of evaporated carbon, and then dissolved, leaving a thin carbon shell which conforms exactly to the original surface (see fig. 1). An electron microscope specimen support grid is first slightly bent, mounted on a $\frac{1}{8}$ in. diameter metal peg and coated with a thick Formvar film. One drop of an aqueous suspension of unfixed spores is then allowed to dry on the film (Fig. 1*a*). The grid, still on the peg, is next transferred to a vacuum plant where a film of carbon about 100Å. thick is deposited on it (Fig. 1*b*). This is done by passing about 40 A. a.c. through the points of two carbon rods lightly pressed together. The intense local heating which occurs at the points causes the carbon to evaporate, and the required deposit is obtained in a few seconds. This carbon film constitutes

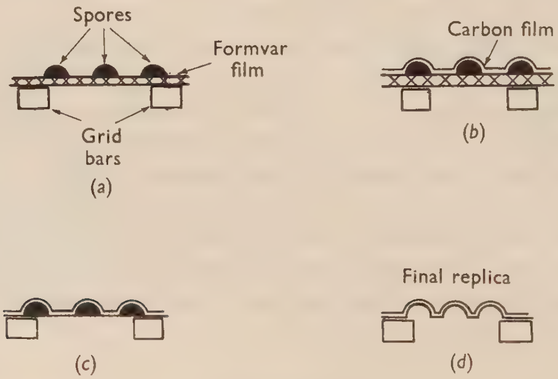

Fig. 1. Diagrammatic representation of the method used for making replicas of spores.

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* Franklin, Williams & Clegg, 1956.

the final replica. The grid and the peg are removed from the plant and the Formvar substrate is washed away by allowing about 1 ml. of chloroform to flow over the grid from a burette with the nozzle held about 2 mm. above the grid (Fig. 1*c*.). The spores can now be dissolved away from the carbon film. This is achieved by placing the grid in an acid mixture which contains 1·5 g. potassium permanganate, 1·5 g. potassium dichromate and 15 ml. of concentrated sulphuric acid. (This mixture is dangerous and should be treated with great care. It should be kept covered at all times as it deteriorates after a few hours; it should therefore be used freshly prepared.) A few drops of the mixture are placed in a small crucible, and the grid is placed, carbon film upwards, on the surface. The spores dissolve in a few minutes, but the carbon film and the copper grid are not attacked (Fig. 1*d*). The grid is removed from the mixture by a pair of forceps and then dipped into water where it is moved about until the acid is washed away. It is then dipped into concentrated hydrochloric acid for a few moments to remove any manganese dioxide formed by the decomposition of the acid mixture. The grid is finally washed in water, dried and shadowed with 60/40 gold-palladium alloy before examination in the electron microscope.

It may be found that the carbon film detaches itself from the grid on immersion in the hydrochloric acid. It will generally remain floating on the surface. A fresh grid or lifter can then be used to transfer it to the water bath, where it is left floating for a short time, before being finally picked up on a grid and dried.

RESULTS

Replicas of thin-walled oval spores of two species, *Bacillus subtilis* and *B. licheniformis*, were examined. The spores of *B. subtilis* were found to be ribbed, the ribbing being in two rather different forms. These are illustrated in Pl. 1, fig. 1, which is of strain 4. In Pl. 1, fig. 1(*a*), a form of spore is shown in which the ribs run longitudinally down the cell; they appear, however, to be joined at the ends. Another form of spore is shown in Pl. 1, fig. 1(*b*). Here, the ribs are in the form of a network on the spore surface. In both cases, the average width of the ribs is about 800 Å, and they appear to be raised from the spore surface by about half this amount. The spores of three other strains of *B. subtilis* (strains 18, 41, 22) were also examined to see whether any difference in the ribbing could be detected. These are illustrated in Pl. 1, figs. 2–4, respectively. It is quite clear that the ribbing in strain 18 is much less marked than in the other two strains. In studying the degree of ribbing, it is important that a large number of spores be examined, since individuals are not always representative. The spores shown are typical of each strain examined.

Spores of three strains of *Bacillus licheniformis* exhibited a different structure (Pl. 2, figs. 5, 6; Pl. 3, figs. 7, 8). The characteristic marking in this species is a longitudinal groove in addition to some ribbing. The groove may perhaps mark a line of weakness on the spore as is suggested in Pl. 3, fig. 7, where one of the spores is broken along it. Pl. 3, fig. 8, shows the rib formation of this strain (93) more clearly. The ribs run lengthwise down the spore and are about 1000 Å. in width, but are not raised as high as those on most of the strains of *B. subtilis*. The spores of the other two strains of *B. licheniformis* (strains 92 and 94) are quite distinct from one another. In the case of strain 92 (Pl. 2, fig. 5) the groove is very shallow, and the ribbing sharply defined, but strain 94 (Pl. 2, fig. 6) has virtually no ribs but a much deeper groove.

The spores of one strain each of *Bacillus brevis*, *B. circulans* and *B. polymyxa* (which form thick-walled oval spores) were examined. Those of *B. brevis* have very little sculpturing on the surface. A single longitudinal rib is shown in several spores in Pl. 3, fig. 9. In some cases it appears to terminate about half way down the spore. Its dimensions are approximately the same as those of the *B. subtilis* spores.

Bacillus circulans spores are covered with a less marked, but rather more complicated, structure. The whole spore has many longitudinal ribs (Pl. 3, fig. 10). The end of one of the spores, visible at the point marked *A* in the figure, is covered with reticulate markings. The width of the ribs is again about 800 Å, although their lateral limits are less clearly defined than in former cases because of the lower angle of slope of the sides.

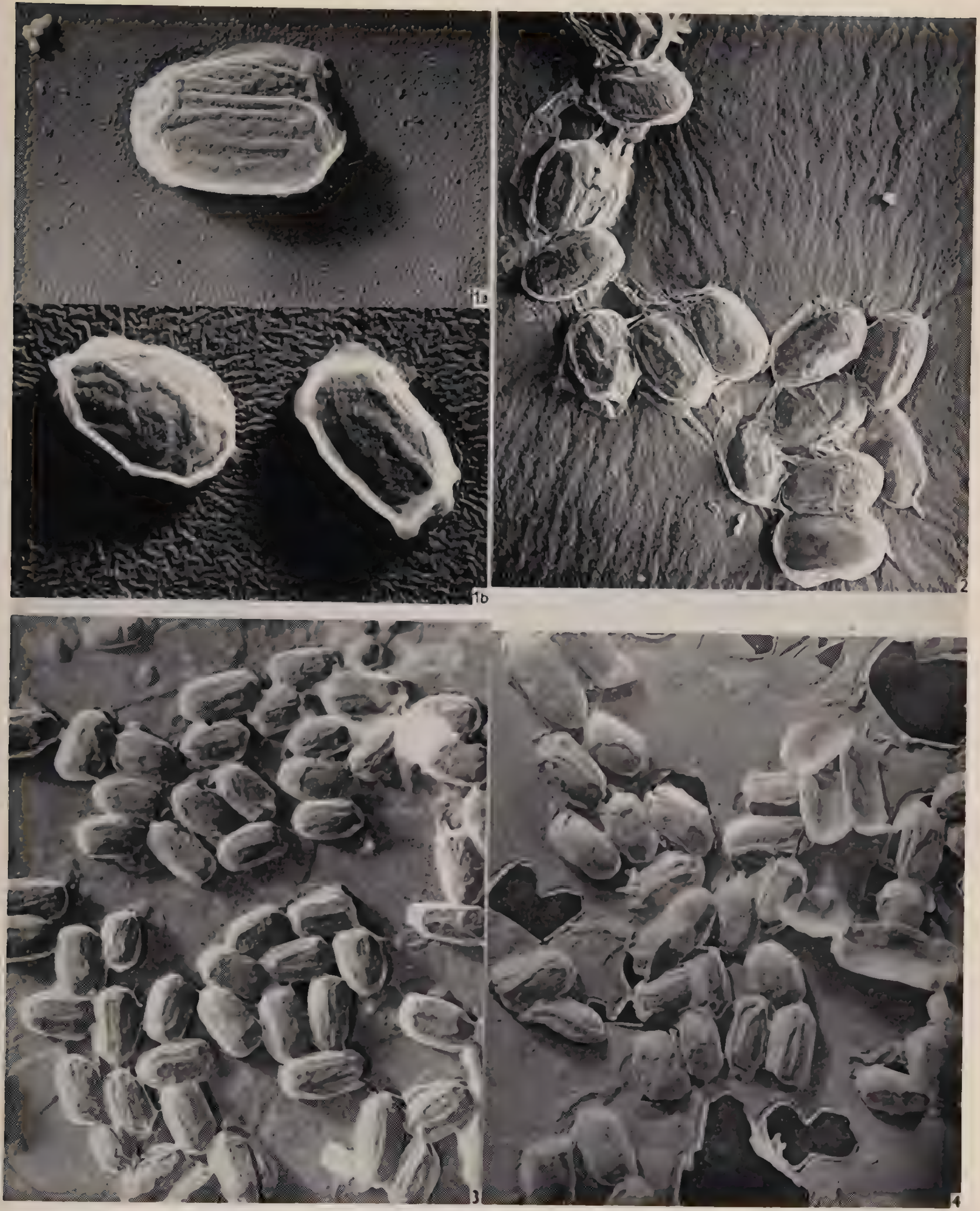
Bacillus polymyxa spores are perhaps the most interesting of those described here. Pl. 4, fig. 11, gives some idea of the appearance of a direct micrograph of the spores. There appear, in some cases, to be a number of terminal spikes (Van den Hoof & Aninga, 1956). An examination of Pl. 4, figs. 12 and 13, which are replicas, shows, however, that this interpretation is false and that the spikes are in fact pronounced ribs seen in profile. These ribs again run lengthwise down the spore and curve round the ends. They are about 1300 Å. wide and raised about the same amount from the spore surface.

The results obtained with thick-walled round spores (*Bacillus sphaericus*), which did not readily separate from the vegetative cell wall are shown in Pl. 4, fig. 14. The cell wall completely masks any surface structure which may be present.

DISCUSSION

The presence of sculpturing on the surface of bacillus spores was quite unexpected. We were not aware of the observations of Meyer (1897, 1903) and those of Van den Hoof & Aninga (1956) appeared after this work was carried out. It is possible that the differences between the spores of the various species so far examined may be sufficiently well defined to be useful in identification. There is little doubt that a set of cultures of these species, whose individual identities were unknown to the operator, could be correctly classified by studying replicas of the spores in the electron microscope.

The difference between strains of two of the species examined here was not so well marked. The basic form of the sculpturing remains, the chief variation being in the number of ribs present and also in the amount by which they are raised above the spore surface. The structural significance of the rib formations is not yet known. It is possible that thin sections may provide some information by comparing the results with micrographs of replicas (Van den Hoof & Aninga, 1956).



D. E. Bradley and D. J. Williams—Morphology of bacillus spores. Plate 1

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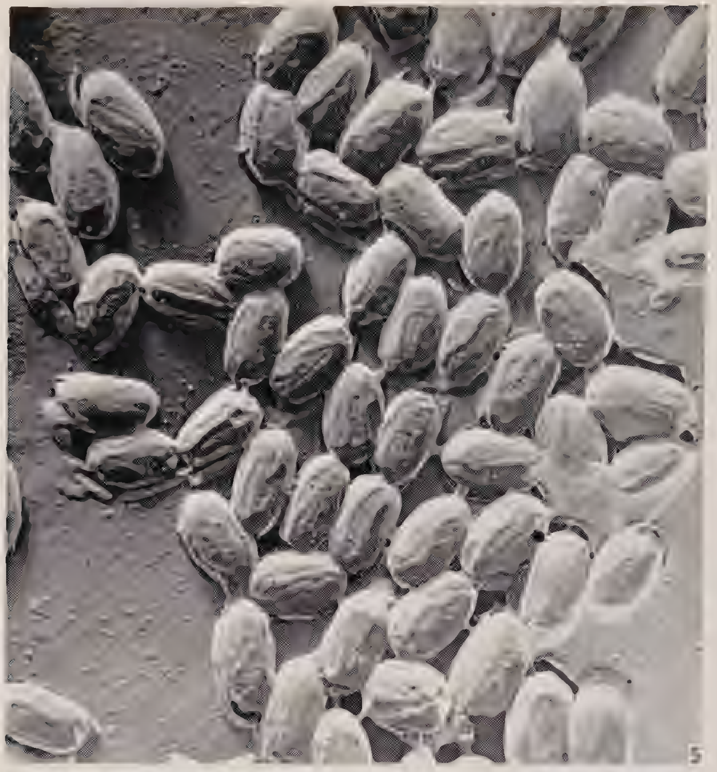

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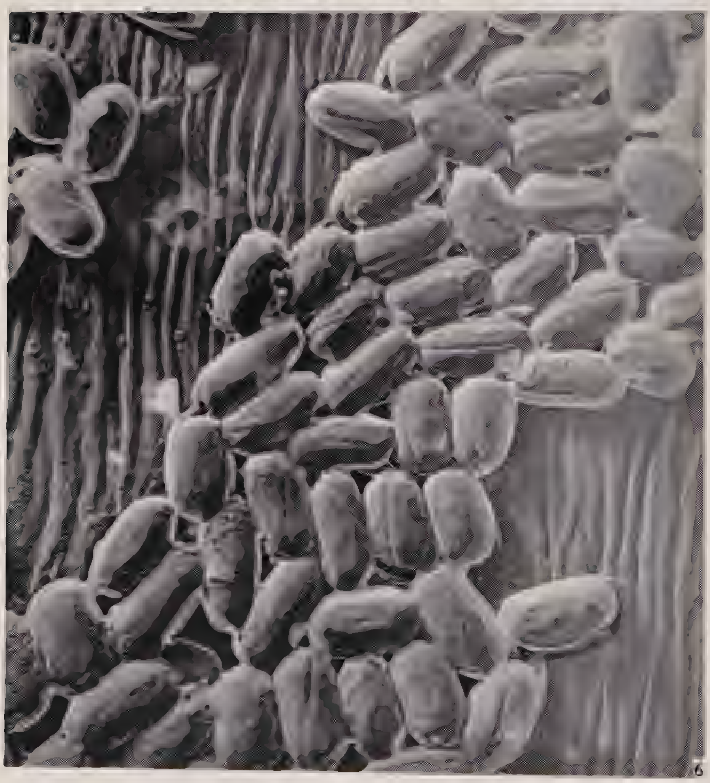

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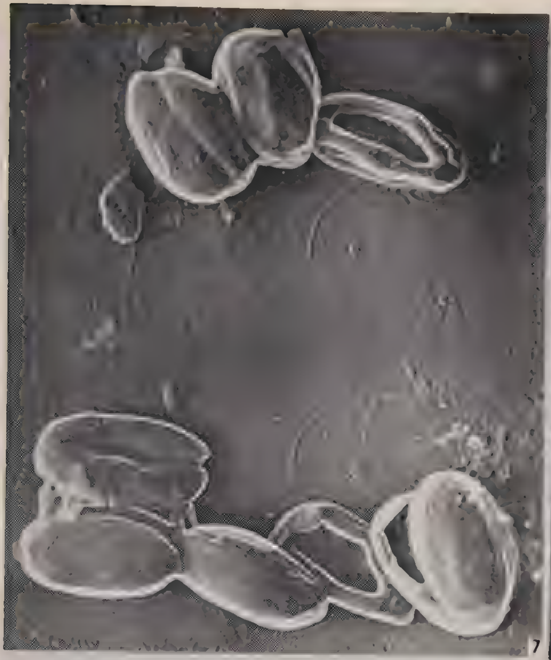
D. E. Bradley and D. J. Williams—Morphology of bacillus spores. Plate 2



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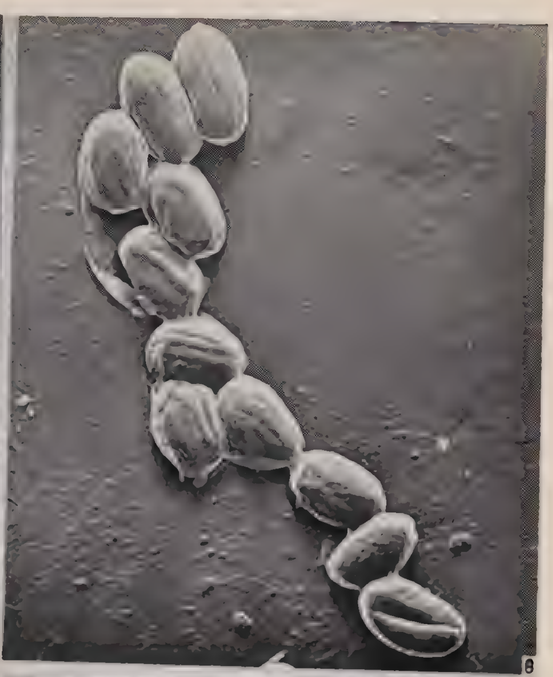

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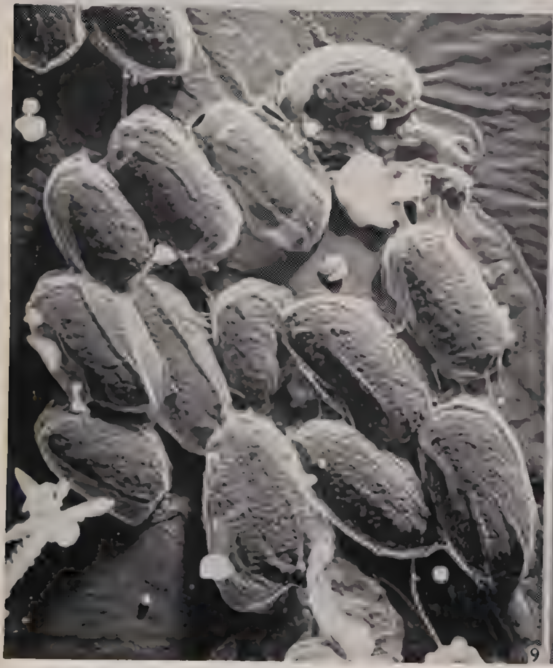

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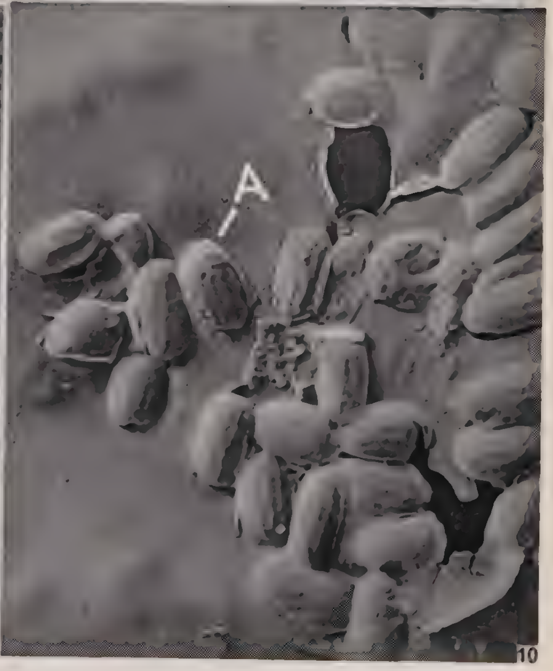

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D. E. Bradley and D. J. Williams—Morphology of bacillus spores. Plate 3

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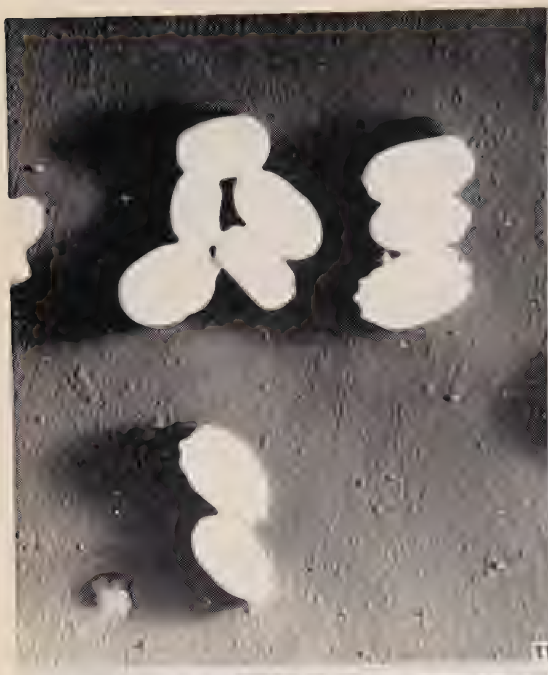
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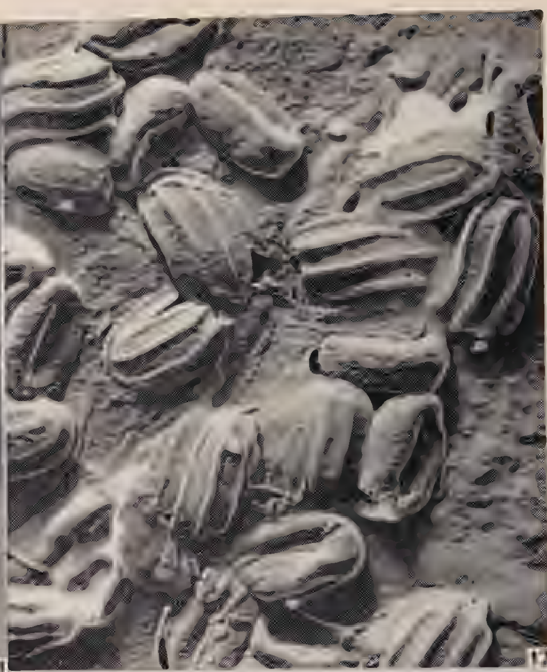
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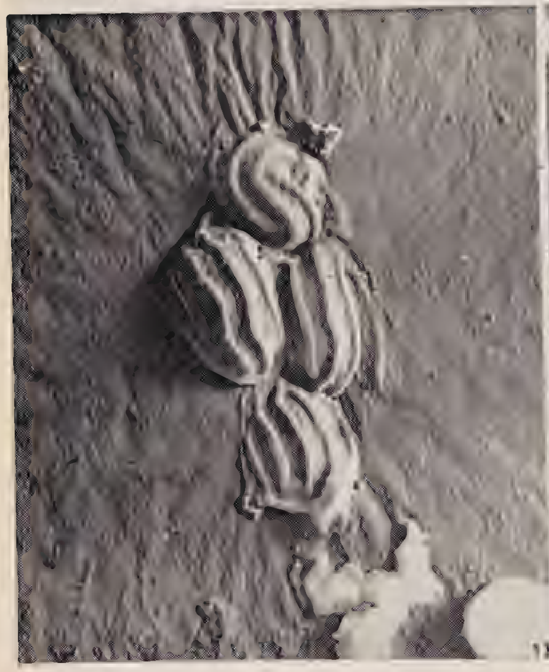
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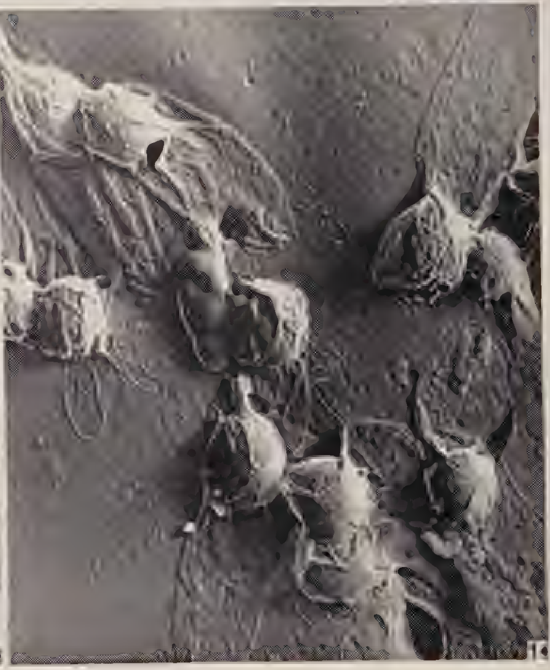
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D. E. BRADLEY AND D. J. WILLIAMS—MORPHOLOGY OF BACILLUS SPORES. PLATE 4

One of the authors (D.E.B.) wishes to thank Dr T. E. Allibone, F.R.S., Director of the Research Laboratory, Associated Electrical Industries Ltd., for permission to publish this paper. The other author (D.J.W.) is indebted to Dr A. T. R. Mattick for the interest he has shown in this work.

REFERENCES

Bradley, D. E. (1954). An evaporated carbon replica technique for use with the electron microscope and its application to the study of photographic grains. *Brit. J. Appl. Phys.* **5**, 96.

Bradley, D. E. (1956). A carbon replica technique for microbiological specimens applied to the study of the division of *Saccharomyces cerevisiae* with the electron microscope. *J. R. micr. Soc.* **75**, 254.

Franklin, J. G., Williams, D. J. & Clegg, L. F. L. (1956). A survey of the number and types of aerobic mesophilic spores in milk before and after commercial sterilization. *J. appl. Bact.* **19**, 46.

Hoof, A. Van den & Aninga, S. (1956). An electron microscope study on the shape of the spores of *Bacillus polymyxa*. *Leeuwenhoek ned. Tijdschr.* **22**, 327.

Knaysi, G., Baker, R. F. & Hillier, J. (1947). A study, with the high-voltage electron microscope of the endospore and life cycle of *Bacillus mycoides*. *J. Bact.* **53**, 525.

Knight, B. C. J. G. & Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.

Meyer, A. (1897). *Flora*, Erg. Bd. **84**, 185.

Meyer, A. (1903). *Practicum der botanischen Bakterienkunde*. Jena: Fischer. (Figure reproduced in Van den Hoof & Aninga, 1956).

Robinow, C. F., (1953). Spore structure as revealed by thin sections. *J. Bact.* **66**, 300.

EXPLANATION OF PLATES

Plate 1

Fig. 1. *Bacillus subtilis*, strain 4. × 12,000. (First published in the *Proc. of* 1*st European Conference on Electron Microscopy, Stockholm*, 1956).

Fig. 2. *B. subtilis*, strain 18. × 7500.

Fig. 3. *B. subtilis*, strain 41. × 4000.

Fig. 4. *B. subtilis*, strain 22. × 4000.

Plate 2

Fig. 5. *B. licheniformis*, strain 92. × 5250.

Fig. 6. *B. licheniformis*, strain 94. × 5250.

Plate 3

Fig. 7. *B. licheniformis*, strain 93. × 10,000.

Fig. 8. *B. licheniformis*, strain 93. × 10,000.

Fig. 9. *B. brevis*. × 9000. (First published in the *Proc. of* 1*st European Conference on Electron Microscopy, Stockholm*, 1956).

Fig. 10. *B. circulans*. × 5000.

Plate 4

Fig. 11. *B. polymyxa*, direct electron micrograph of shadowed spores. × 5000.

Fig. 12. *B. polymyxa*, carbon replica of spores. × 5000.

Fig. 13. *B. polymyxa*. × 5000 (By permission of *Research*.)

Fig. 14. *B. sphaericus*. × 5250.

(*Received* 21 *January* 1957)

Bawden, F. C. & Pirie, N. W. (1957). *J. gen. Microbiol.* 17, 80–95

The Activity of Fragmented and Reassembled Tobacco Mosaic Virus

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SUMMARY: Studies of the products obtained when tobacco mosaic virus (TMV) is disrupted with alkali or phenol suggest that immunological specificity is primarily an attribute of the protein and infectivity of the nucleic acid. Although exposing the virus to alkali produces infective fragments, it also causes much inactivation, and much of the nucleoprotein sedimented when preparations are ultracentrifuged at pH 6 is not infective. The unsedimented protein fragments are inhibitors of infection; from such unsedimentable material, which at 5 g./l. produced no lesions when inoculated to *Nicotiana glutinosa*, some infective nucleoprotein could sometimes be separated by precipitation with ammonium sulphate, followed by ultracentrifugation. The infectivity of fragmented TMV is ephemeral, but it is stabilized when the fragments are reunited. Nucleic acid preparations made by phenol are quickly inactivated by ribonucleases from pancreas or leaves; pancreatic ribonuclease also inactivates alkali-made fragments, but the infectivity of these is stabilized by leaf ribonuclease. Phenol-made preparations are much less infective per unit of phosphorus than intact TMV, but measurements of the relative infectivities of the two kinds of inocula are complicated because the two respond differently to dilution and they are not equally able to infect *N. glutinosa* leaves in different physiological states. Although urea does not inactivate phenol-made preparations, nucleic acid made by exposing TMV to urea has little or no infectivity. The possibility that infective TMV can be reassembled *in vitro* from previously non-infective components cannot be excluded, but all the results that could be interpreted as suggesting this are also interpretable in other ways, either by the removal of inhibitors of infection or by the stabilization of infective fragments that otherwise would have become inactive before testing.

The significance of the various types of anomalous particles that occur in extracts from plants infected with tobacco mosaic virus (TMV) is undecided and it has also remained uncertain how the property of infectivity is distributed among them. Preparations that contain the predominant type of particle, a rod 15 mμ. wide and about 300 mμ. long, are more infective than preparations of the smaller particles, but this does not justify the almost generally accepted conclusion that such rods are the minimum infective units, are uniformly infective and the only biologically important particles. Indeed there has long been some evidence to the contrary. Not only did X-ray analysis show that the rods are made from subunits (Bawden, Pirie, Bernal & Fankuchen, 1936), but rods were produced *in vitro* by aggregating small particles (Bawden & Pirie, 1945). Also, the fact that filters with pores of 50 mμ. diameter gave infective filtrates from sap, whereas membranes with much larger pores failed to do so from purified virus preparations, suggested that sap might contain smaller infective units (Bawden & Pirie, 1937). Furthermore, although rapidly sedimenting particles provided the most infective preparations, slowly sedimenting nucleoprotein had some infectivity but no demonstrable rods even approaching 300 mμ. in. length (Bawden & Pirie, 1945; Crook & Sheffield, 1946). However, occasional rods are not readily detectable in the presence of many particles of other sizes, especially in unshadowed electron micrographs, and the small infectivity was plausibly explained by postulating contamination with a few 300 mμ. rods.

Similarly, it was plausible to assume that the infectivity remaining in TMV preparations disrupted by alkali (Best, 1936; Bawden & Pirie, 1940*a*; Schramm, 1943) was not a feature of the small particles but of remaining intact rods. Alkali and other disrupting agents, such as sodium dodecyl sulphate (Sreenivasaya & Pirie, 1938) and phenol (Bawden & Pirie, 1940*b*), decreased infectivity so rapidly that there was no call to regard them as anything except inactivators. However, recent work with disrupted preparations, stimulated by the current interest in the variety of particles produced when TMV multiplies and in nucleic acids as carriers of specific activities, has established the ability of such agents to produce smaller infective units. Not only can preparations disrupted by sodium dodecyl sulphate (Fraenkel-Conrat, 1956) or phenol (Gierer & Schramm, 1956) be infective when free from intact rods 300 mμ. long, but the infectivity is now clearly associated with less stable particles, for it is lost in a day or so at 18° and very rapidly in the presence of ribonuclease.

Most treatments that separate TMV into protein and nucleic acid denature the protein, but exposure to glycine buffer at about pH 10·5 decreases infectivity while leaving the preparation soluble and still reacting specifically with TMV antiserum (Bawden & Pirie, 1940*b*). This treatment produces a range of products with different properties (Schramm, Schumacher & Zillig, 1955). Some of the residual infectivity may still be in intact particles and most of it is removed by ultracentrifugation, but a part is associated with smaller particles (Schramm *et al.* 1955; Fraenkel-Conrat & Williams, 1955). The great loss of infectivity, however, shows that much of the easily sedimentable material with the gross structure and composition of TMV particles is no longer infective. The unsedimentable protein is mostly free from nucleic acid and there is no evidence that this is infective.

Various treatments aggregate the protein fragments produced from TMV by alkali (Schramm, 1943), as they also do the small particles present in extracts from infected plants (Bawden & Pirie, 1945, 1956*a*; Takahashi & Ishii, 1952, 1953; Commoner, Newmark & Rodenberg, 1952; Jeener & Lemoine, 1953), and produce rods resembling usual TMV particles. When the proteins are aggregated in solutions containing nucleic acid, whether from TMV (Fraenkel-Conrat, 1956) or other sources (Hart & Smith, 1956), nucleic acid is incorporated in the rods. The 'reconstitution' of particles with the form and gross chemical composition of TMV has been stated to confer infectivity on mixtures of the two components, neither of which previously possessed it (Fraenkel-Conrat & Williams, 1955; Lippincott & Commoner, 1956; Siegel, Wildman & Ginoza, 1956). The infectivity of the reconstituted preparations is slight, and in none of the quoted papers is there unequivocal evidence that infective particles were produced from non-infective components. Alternative interpretations have already been advanced by Lippincott & Commoner (1956) and by Bawden & Pirie, (1956*b*); these include: first, the possibility that the treatments which separated aggregated particles from the preparation removed already existing infective material from an environment in which its infectivity was obscured by an inhibitor of infection; secondly, the possibility that aggregation stabilized material which otherwise would have lost its infectivity during the manipulations before and during inoculation.

Commoner, Lippincott, Shearer, Richman & Wu (1956) described conditions that yielded preparations of recombined nucleic acid and protein with much greater infectivities than those previously reported. Some fractions from their preparations had 40 % of the infectivity of the starting material, but the significance of this is difficult to assess, because they also showed that their starting material was heterogeneous with respect to infectivity, as probably are our TMV preparations and most others. Their most infective 'reconstituted' particles, therefore, still carried only a fraction of the maximum possible infectivity.

Inhibitors of infection are usually not difficult to recognize, but it may be less easy to distinguish between stabilization of infectivity and the activation of previously non-infective materials. Thus, if a nucleic acid preparation were so labile that its infectivity was destroyed on the surface of a leaf, or as it entered a cell, and if combination with protein protected it against this inactivation, the result would be operationally indistinguishable from creating infectivity. This illustration could be more than a hypothetical analogy, because leaves contain ribonuclease, which has no permanent effect on intact TMV *in vitro*, but which rapidly inactivates nucleic acid preparations made by phenol.

The experiments we describe below were made to find how biological activity is distributed among the components when TMV is disrupted in various ways, and to distinguish between alternative explanations for apparent gains in infectivity when the nucleic acid and protein are recombined. The results confirm that fragments of the rods can be infective and they amply demonstrate the difficulties of drawing firm conclusions from infectivity tests when inocula differ in composition or have been exposed to different conditions.

METHODS

Most of our experiments were made with the type strain of TMV propagated in tobacco (*Nicotiana tabacum* L., var. White Burley), but a strain that infects leguminous plants systemically (Bawden, 1956) was used in some, and this was propagated in French beans (*Phaseolus vulgaris*, var. Prince). All of the several different preparations of TMV used were made by precipitation with ammonium sulphate and at pH 3·3, followed by incubation with citrate and sedimentation in the ultracentrifuge. They were in the state defined by Pirie (1956) as TMV (L), which means they were highly aggregated and free from many of the components contained in less drastically fractionated preparations, but still with just detectable ribonuclease activity.

Infectivity tests were made by the local-lesion method, using *Nicotiana glutinosa* as the host. Usually 8 half-leaves were inoculated with each sample being tested, and the different inocula were distributed over the test plants so that each occurred equally often on any one plant, on left- and right-hand half-leaves, and at each leaf position. Serological tests were made by titrating antigen solutions against TMV antiserum at a constant concentration, using methods already described (Bawden & Pirie, 1956*a*).

Leaf ribonuclease was made from peas (*Pisum sativum* L.) as described by Holden & Pirie (1955*a*, *b*) and its activity is expressed in the unit defined by them. When assayed against yeast nucleic acid, during the first stages of hydrolysis, 1 unit is equivalent to 1 μg. commercial pancreatic ribonuclease (Armour 'crystalline').

RESULTS

Fission by alkali

Between pH 9·5 and 10·5 solutions containing 10 g./l. TMV soon lose their opalescence and anisotropy of flow when kept at 0 to 5°. At pH 10 these characters are still perceptible after 10 but not after 30 min. With the change in optical characters of the preparation, there is also a change in the precipitation behaviour with antiserum. Flocculation occurs more slowly and, with antiserum diluted 1/100 and antigen solutions decreasing from 1 g./l., there is now usually a zone of antigen excess in which precipitation is inhibited; the type of floccules also changes from the fluffy open type, typical of flagellar (H-type) antigens, to denser granules that settle to a compact sediment, more resembling precipitates given by somatic (O-type) antigens. The precipitation end-point also diminishes and the whole effect is to change purified TMV preparations so that they behave serologically like the small particles of anomalous protein present in infective sap. The effect is reversible and the serological behaviour reverts to the flagellar type when the alkali-treated preparations are aggregated by exposure to ammonium sulphate or acid.

After a few hours at pH 10·5, the serological behaviour changes little, but infectivity continues to decrease during the next few days. Table 1 shows that the decrease is rapid at first and then becomes increasingly slow. After 100 hr. at 0°, the preparation used in the experiment was brought to pH 6·3 and ultracentrifuged for 1 hr. at 70,000 *g*; inoculated at 32 mg./l. to *Nicotiana glutinosa* the sedimented material produced lesions but the unsedimented material did not. We have similarly centrifuged many TMV preparations after they were exposed to pH 10–10·5 for periods between 1 and 100 hr. and despite large differences in the amount of material that sedimented, it always contained 0·5–0·6 % P and had the usual physical properties of TMV. Its infectivity was much decreased, usually to 1–5 % that of the starting virus preparation, showing that particles with the gross morphology of TMV and similar nucleic acid content can differ greatly in their ability to infect plants.

The material remaining in the supernatant fluids after ultracentrifugation at about pH 6 was usually infective when the exposure to alkali was restricted to a few hours but not when it extended to a few days. The infectivity of these fluids has varied from 0·1–1·0 % of that of TMV preparations containing the same amounts of P, and it seems not to be associated with residual TMV rods that failed to sediment, for none could be seen in electron micrographs. Also, when enough control TMV was added to such fluids to double their infectivity, the added virus was readily removed by ultracentrifugation. Further, as described later, these fluids lose their infectivity in conditions where normal TMV does not.

Table 1. *Effect of alkali on infectivity of TMV*

Samples were taken at intervals from a 10 g./l. TMV preparation kept at pH 10 and 0°, brought to pH 7 and kept at 0° until the end of the experiment when they were inoculated at the stated concentrations to the same batch of *Nicotiana glutinosa*. After 100 hr. at pH 10, the preparation was brought to pH 6·3, centrifuged for 1 hr. at 70,000 rev./min., and the material in the supernatant fluid and pellet used as separate inocula. The numbers of lesions are the totals on 6 half-leaves.

Duration of exposure at pH 10	Concentration of inoculum (mg./l.)	Numbers of lesions
0 min.	2	233
5 min.	2	48
40 min.	4	72
4 hr.	8	69
10 hr.	16	96
22 hr.	32	94
46 hr.	32	42
70 hr.	32	26
Material unsedimented at pH 6·3 after 100 hr. at pH 10	32	0
Material sedimented at pH 6·3 after 100 hr. at pH 10	32	32

The infectivity of the supernatant fluids depends not only on the length of exposure to alkali, but also on the pH value at which the fluids are ultracentrifuged; it is greater at pH values between 7 and 10 than at 6. When supernatant fluids from preparations already ultracentrifuged at pH 9 are brought to pH 6 and ultracentrifuged again, the greater part of the infectivity is now in sedimentable material. At different pH values there seems to be a different equilibrium between forces disrupting TMV rods and forces causing the parts to reassociate, and whether the rods reformed when the pH value is brought to 6 are infective or not possibly depends on whether or not a component fragment was inactivated during the disruption or while free in the alkali.

Ultracentrifugation at pH 6, particularly after prolonged exposure to pH 10 or after standing for some days at pH 6, gives supernatant fluids containing material that produces no lesions in *Nicotiana glutinosa*, even when inoculated at 1–10 g./l. This material, however, is far from inert. It still reacts specifically with TMV antiserum, although it does so only over a restricted zone of antigen/antiserum ratios. It also inhibits infection by TMV, a fact of special importance because such materials are used in experiments on the ‘reconstitution’ of the virus. Fraenkel-Conrat & Williams (1955) stated that their proteins made in this way did not inhibit infection, but the manner of their test would not show the effect. Their proteins and TMV were mixed concentrated and then the solutions were diluted before they were inoculated to test plants, and it is a characteristic of inhibitors that their action is abolished by dilution (Bawden, 1954). Table 2 shows the extent to which two preparations of such material decreased the numbers of lesions produced by purified TMV. When mixed with preparations of infective nucleic acid made by disrupting TMV with phenol, these proteins also decrease the numbers of lesions produced.

Table 2. *TMV protein as an inhibitor of infection*

Both TMV protein preparations were the material that remained in the supernatant fluids when TMV preparations were ultracentrifuged at pH 6·3 after spending some days at pH 10 and 0°. The protein and TMV were mixed immediately before inoculation, and the numbers of lesions are the totals produced on 8 half-leaves.

	Numbers of lesions	
Inoculum	Protein 1	Protein 2
Protein 5 g./l.	0	0
Protein 5 g./l., TMV 1 mg./l.	18	44
Protein 1 g./l., TMV 1 mg./l.	79	117
Protein 0·2 g./l., TMV 1 mg./l.	140	155
TMV 1 mg./l.	161	185

The fact that alkali-produced fragments of TMV inhibit infection when present in inocula at concentrations greater than about 0·5 g./l. makes infectivity tests with such inocula of doubtful significance. So, too, it greatly complicates the interpretation of tests on the 'reconstitution' of infective virus from apparently non-infective components, for these components might contain material with its intrinsic infectivity obscured. From the supernatant fluids of ultracentrifuged, alkali-treated preparations of TMV, which gave no lesions at 5 g./l., we have often separated infective material by again ultracentrifuging after the preparations were precipitated with ammonium sulphate and redissolved. The small amount of sedimented material had, weight for weight, about 1/100 the infectivity of control TMV. Our treatment may have produced infective particles by combining previously non-infective components, but the conclusion does not follow of necessity, for the sedimented fraction itself manifested no infectivity when, instead of in water, it was taken up in the non-infective fluid from which it was derived. It is at least as likely that the starting fluid contained infective material and that the treatment merely made possible its removal from the inhibiting action of other materials.

Fission by phenol

The solutions produced by disrupting TMV preparations with alkali contain both protein and nucleic acid. Exposure to phenol denatures protein, and Bawden & Pirie (1940*b*) found that 20 hr. in 40 g./l. phenol at 23° destroyed both serological specificity and infectivity. Gierer & Schramm (1956) found that more concentrated phenol used for shorter times at 0° destroyed serological specificity but not all the infectivity. We have confirmed this and our technique for disrupting TMV with phenol is based on theirs.

The concentration of TMV is not critical, but the ratio of water to phenol and the salt concentration both are. Essentially the method involves the partition of denatured protein and nucleic acid between the phenol layer and the water layer; the nucleic acid remains predominantly in the water layer only when the salt concentration is below 0·04 M.

Equal volumes of 900 g./l. aqueous phenol and 5–25 g./l. TMV in 0·017 M-sodium citrate were mixed at 0°, shaken briskly by hand for a few seconds and centrifuged in a tube surrounded by ice for 15 min. at 3000 rev./min. The supernatant fluid was siphoned off, again shaken with a quarter of its volume of the aqueous phenol, and centrifuged in the same way. The supernatant fluid was now shaken twice for a few minutes with 10 vol. of sodium-dried ether to remove most of the phenol and then, still at 0°, the ether was removed by exhaustion with a filter pump while the fluid was rocked gently. The solution contains about half the nucleic acid of the starting TMV and more can be separated by shaking the combined phenol layers with their own volume of water and repeating the centrifugation and extraction with ether.

The infectivity of freshly made preparations is, as Gierer & Schramm (1956) observed, about that of TMV solutions containing 1/1000 the concentration of P, but relative infectivities of the two types of inocula are difficult to express in such terms for they depend on the plants inoculated and on the concentration of the inocula. No rods can be seen in electron micrographs, and this failure is more significant than with alkali-treated TMV, which contains much confusing protein. Rods are readily apparent when TMV is added in amounts needed to give less than the observed infectivity. Also, no pellet forms when the fluids are ultracentrifuged, though one does when TMV is added in amounts to give the observed infectivity. Furthermore, the fluids have consistently failed to give a precipitate with TMV antiserum, even when their infectivity was 10 or more times that of TMV preparations diluted to their precipitation end-point. At 80,000 *g* the infectivity concentrates towards the bottoms of the centrifuge tubes, and electron micrographs show narrow filaments, often intertwined to give a mesh-like appearance, with a size and shape compatible with the slow sedimentation.

After dialysis such preparations consist predominantly of nucleic acid, which is presumably what is resolved by the electron microscope; the preparations give the characteristic nucleic acid absorption curve and negative results in colour tests for protein. Electrophoretic examination shows a single small peak, with a mobility in M/15 pH 7 phosphate buffer of $17{\cdot}5 \times 10^{-5}$ cm^2 sec^{-1} v^{-1}, a value consistent with the component being nucleic acid. Gierer & Schramm (1956) have argued that the nucleic acid alone is infective, and we have no evidence to conflict with this conclusion. Absorption spectrography and protein tests do not exclude the possibility that the infectivity of the preparations resides in protein, because the amount necessary if it had the infectivity of normal TMV would be undetectable by these methods; that it does not reside in intact TMV rods is demonstrated by electron micrography, ultracentrifugation and serological tests.

Contamination of the nucleic acid preparations by residual TMV is also excluded by the lability of their infectivity; at 18° they often lose their infectivity within a day, whereas TMV is stable indefinitely. The infectivity also differs qualitatively from that of normal TMV both in its behaviour towards test plants and in its response to dilution. Table 3 compares the two types of preparation at three concentrations and on two lots of plants, one young, with the fragile, light-green leaves typical of 'shade-grown' plants, and the other older, with tougher and dark-green leaves. The relative infectivity of the two preparations obviously depends on the state of the leaves inoculated and on the concentrations at which they are compared. The decrease in infectivity on dilution is greater with the old than with the young plants, but with both lots of plants the decrease is steeper with the nucleic acid preparation. Indeed, the experiment recorded in Table 3 gave a less rapid decrease than usual. To quote a more typical result with highly susceptible plants, when TMV at 32, 8, 2, 0·5, and 0·12 mg./l. gave 1120, 439, 135, 62 and 25 lesions, a nucleic acid preparation diluted 1/20, 1/80, 1/320, 1/1280 and 1/5120 gave 469, 100, 18, 4 and 0 respectively. With inocula of the two types that give similar average numbers of lesions per half-leaf when inoculated to plants with 8–10 well-developed leaves, the numbers produced on individual leaves by the nucleic acid varies much more than with a normal TMV preparation. Also, on such plants TMV usually produces more lesions on leaves on the lower half of the stem, whereas the nucleic acid preparations produce more on the younger leaves, particularly when the lower ones are beginning to 'harden'.

Table 3. *Effect of age of plant and dilution on relative infectivity of TMV and nucleic acid preparation*

	Numbers of lesions on	
Inoculum	Young plants	Old plants
TMV 50 mg./l.	560	1240
10 mg./l.	353	460
2 mg./l.	185	146
Nucleic acid diluted 1/4	344	60
1/20	163	23
1/100	65	4

The local lesions produced in *Nicotiana glutinosa* by the nucleic acid preparations are of the same type as those produced by the virus preparation from which they are derived and they become visible and countable at about the same time. A strain of TMV, which becomes systemic in leguminous plants and when obtained from them produces a characteristic type of lesion in *N. glutinosa* (Bawden, 1956), was used in a few tests. When a purified preparation from French beans was treated with phenol, the resulting nucleic acid preparation was infective and the lesions it produced resembled those caused by the parent virus, being much smaller than, and taking twice as long to appear as, those caused by TMV. We have also inoculated tobacco plants with nucleic acid prepared from the type strain of TMV without finding any change in the type of systemic symptoms caused. Some plants inoculated as young seedlings in the winter were much stunted and had their leaves acutely deformed as reported by Commoner *et al.* (1956) with some of their 'reconstituted' viruses, but so too did comparable plants inoculated at the same time with the TMV from which the nucleic acid was derived.

Fission by urea

The infectivity of the best preparations of the water-soluble products from phenol-treated TMV is much too great to be attributed to contaminations with unaffected virus particles, but it is still too small to be identified certainly with the nucleic acid rather than with some other minor component of the preparations. In an attempt to get more active preparations, which might settle this problem, we tried various other treatments for disrupting the virus. None succeeded in its prime object, but tests with urea demonstrated an interesting fact.

TMV is disrupted and loses infectivity when exposed to neutral or slightly alkaline 5–8 M solutions of urea (Bawden & Pirie, 1940*a*). The protein, which stays dissolved so long as the urea is concentrated, precipitates on dilution in the presence of some salt, while the nucleic acid remains in solution. When fission is incomplete, either because the exposure is too brief or the TMV too concentrated, some virus remains in solution with the nucleic acid. This can be separated by ultracentrifugation and has the same infectivity, weight for weight, as the starting preparation; hence urea seems not to produce sedimentable, non-infective nucleoprotein such as occurs after exposure to alkali. The unsedimented nucleic acid produced very few lesions, and with the idea that infectivity might have been destroyed by oxidation, many variations of the treatment were tried; urea was used in the presence of cysteine, azide, ascorbic acid or thioglycollate, and guanidine or thiourea were used instead of urea, but none gave preparations which, after ultracentrifugation, possessed significant amounts of infectivity.

An obvious explanation for this would be that structures essential for nucleic acid preparations to be infective are destroyed by urea, but this interpretation does not fit well with the fact that preparations made with phenol are not inactivated when later exposed to 8 M-urea. Hence it seems that, either the process of separating the nucleic acid by urea damages it in some way avoided by phenol, or infectivity is carried by something that remains in the protein coagulum produced by urea. Disruption of TMV with strontium nitrate (Pirie, 1954) also yielded nucleic acid preparations with too little infectivity to be significant.

Effects of ribonuclease

Gierer & Schramm (1956) reported that pancreatic ribonuclease inactivated nucleic acid preparations made by phenol from TMV; we have confirmed this and also found that they are inactivated by leaf ribonuclease. Infectivity falls in a systematic way, depending on the amount of enzyme present and on the time of exposure to it. Table 4 shows that 2 hr. at 18° with 0·002 unit enzyme/ml. removes almost all the infectivity. This exposure does not affect the precipitability of the nucleic acid by acid. After 2 hr. with 0·02 unit enzyme/ml., no soluble P is detectable in preparations treated with uranyl:trichloroacetic reagent (Holden & Pirie, 1955*a*) and, by this criterion, with 0·2 unit enzyme/ml. only 10 % is hydrolysed in 2 hr. If, then, as seems likely, ribonuclease action is responsible for the loss of infectivity, the loss accompanies the first stages of the action rather than total hydrolysis of the nucleic acid.

Table 4. *Inactivation by ribonucleases of nucleic acid prepared by phenol treatment of TMV*

The infectivity is expressed as the total numbers of lesions formed on 8 half-leaves. LRase = leaf ribonuclease and PanRase = pancreatic ribonuclease.

Treatment of phenol-made nucleic acid before use as inoculum	Infectivity
Kept at −20°	382
2 hr. at 18° alone	310
Mixed with 2 units/l. LRase just before testing	240
7 min at 18° with 0·2 unit/l. LRase	240
2 hr. at 18° with 0·2 unit/l. LRase	29
2 hr. at 18° with 2 units/l. LRase	7
2 hr. at 18° with 3 mg./l. PanRase	0

At concentrations above about 5 mg./l. pancreatic ribonuclease strongly inhibits infection of *Nicotiana glutinosa* by TMV, but exposure to the enzyme has no permanent effect on the virus, which can be recovered unimpaired from non-infective mixtures (Loring, 1942; Kleczkowski, 1946). Preparations of the leaf ribonuclease also inhibit infections by TMV, though much less strongly per unit of enzyme activity than the pancreatic enzyme. Inhibition is not apparent until there are about 25 units enzyme/ml., which is 1000 times as much as was used to destroy the infectivity of the nucleic acid preparation used for the experiment reported in Table 4. When infective nucleic acid preparations are mixed with leaf enzyme at 5 units/ml., or with pancreatic ribonuclease at 1 mg./l. and immediately inoculated to *N. glutinosa*, no lesions are produced. With such a susceptible substrate it is operationally impossible to decide whether this is because the enzyme preparations inhibit infection or because they immediately destroy infectivity.

Although both types of ribonuclease have similar effects on phenol-made products of TMV, they affect alkali-made products differently. Table 5 shows that pancreatic ribonuclease destroyed the infectivity of both types of product; leaf ribonuclease destroyed infectivity of the phenol-made ones only, and the alkali-made one retained more of its infectivity when exposed to this enzyme than when alone at 18°. The presence of leaf ribonuclease never stabilized the infectivity of alkali-made products completely, and experiments in which the enzyme preparations were added to products already partially inactivated by exposure to 18° do not suggest that its action should be interpreted as the synthesis of infective particles from previously non-infective components.

Inactivation that otherwise would have occurred seems to be prevented. It is, though, easy to get the appearance of synthesis unless unchanged starting material is included in tests comparing relative infectivities of fluid at the end of an experiment. We have done this by keeping samples of the starting material frozen at −20°, a condition in which both the alkali- and phenol-made products retain infectivity apparently unimpaired for long periods.

Table 5. *The effects of leaf and pancreatic ribonuclease on infective products made from TMV with alkali or phenol*

Aliquots (0·2 ml.) of the two types of infective product were mixed with 0·1 ml. of either 0·02 M-sodium citrate (pH 6) or citrate containing either 0·1 units of leaf ribonuclease (LRase) or 0·1 μg. of pancreatic ribonuclease (PanRase), and the mixtures were diluted with 0·7 ml. of water before inoculation to *Nicotiana glutinosa*.

	Numbers of lesions	
Treatment of product	Alkali product	Phenol product
Kept frozen	166	363
20 hr. at 18° alone	4	14
2·5 hr. at 18° alone	74	154
2·5 hr. at 18° with LRase	72	0
2·5 hr. at 18° with PanRase	0	0
7 hr. at 18° alone	21	108
7 hr. at 18° with LRase	80	0
7 hr. at 18° with PanRase	0	0

Comparable controls seem not to have been kept in other experiments that have reported the synthesis of infective particles, and some of the reports may therefore refer to stabilization of infectivity rather than to synthesis. The stabilization of infectivity in alkali-made products by preparations of the leaf enzyme may have nothing to do with ribonuclease activity, for these preparations probably contain much more extraneous material than do preparations of the pancreatic enzyme. Experiments with other proteins, and with preparations of leaf ribonuclease after exposure to various types of inactivation, will be needed before any conclusions can be drawn on the part played in the stabilization by the enzyme itself.

Some of the ribonuclease activity present in purified preparations of TMV is carried into the fission products made by alkali. When these products are incubated at 37° in pH 6 0·01 M-citrate, about half the P becomes unprecipitable with uranyl:trichloroacetic acid in 8 hr. and all after 24 hr. The protein also denatures and coagulates during this treatment. When yeast nucleic acid is added to such incubated preparations, it also is hydrolysed, but less rapidly than the virus nucleic acid. This resembles the behaviour of TMV treated with trichloroacetic acid (Pirie, 1956), for there also the enzyme attacked virus nucleic acid more readily than yeast nucleic acid. Another similarity is that hydrolysis is more rapid with citrate than with maleate buffer, which is a feature of the enzyme's action on nucleic acid that is linked with protein (Pirie, 1957), and the hydrolysis, like that by ribonuclease obtained from pea plants, is inhibited by zinc.

The ribonuclease activity of our preparations made by disrupting TMV preparations with phenol is so low as to be equivocal when tested for by our methods, and it is uncertain to what extent the 'depolymerization' reported by Gierer & Schramm (1956) to accompany the loss of infectivity is a consequence of their products containing some ribonuclease. That such contaminations may in part determine the stability of the nucleic acid preparations was suggested by experiments in which we added leaf ribonuclease to one sample of TMV and yeast nucleic acid to another before disrupting with phenol. When the resulting products were exposed to 18°, the one made from TMV plus ribonuclease lost infectivity more rapidly, and the one from TMV plus yeast nucleic acid, less rapidly than the one from TMV alone.

As TMV is not permanently affected by exposure to ribonuclease *in vitro* and the disrupted preparations are inactivated so readily, it is tempting to correlate this difference with the small infectivity of the fragments. All leaves so far examined contain ribonuclease, and a susceptible substrate might well have difficulty in moving unharmed from where it encounters the leaf to the site where it can become established and effective. We have compared the ribonuclease activity of leaves from plants of different ages, and from different positions on the stem, but have not found differences of the same order as those between their relative susceptibility to infection by TMV and phenol-made derivatives. Also, we have added yeast nucleic acid and zinc sulphate to inocula, with the idea that these might protect TMV fragments from attack by ribonuclease, but the additions did not increase the numbers of lesions produced. These negative results are inconclusive, and it is not easy to see how positive evidence can be got for the idea that the poor infectivity of freshly fragmented virus is not intrinsic to it but a consequence of its sensitivity to a host-cell enzyme.

DISCUSSION

Work in several laboratories has shown that fragmented preparations of TMV can be infective, and that their infectivity is too ephemeral to be conferred by contaminations with unchanged virus particles. It is also well established that these fragments can re-associate and produce particles with the form and constitution of the original TMV; treatments that re-associate fragments stabilize the infectivity and often allow infective material to be separated from preparations which previously seemed to lack this character. These facts raise four obvious questions: have the infective fragments any biological significance? can the association of non-infective fragments confer infectivity on a reconstituted particle? what is the chemical nature of the infective fragments? and what change leads to their loss of infectivity?

The small protein and nucleoprotein fragments produced by disrupting TMV with alkali have obvious similarities with the anomalous particles that fail to sediment or sediment only slowly when infective sap is ultracentrifuged. Although these anomalous particles are serologically distinguishable from most of the proteins produced from TMV by alkali (Kleczkowski, 1957), both react with TMV antiserum in the same qualitative manner and both can be reassembled into the characteristic rods. Alkali, then, might be reversing the process whereby virus particles are synthesized from intermediaries, or duplicating some process in cells whereby particles are naturally disrupted. Evidence from experiments with leaves supplied with $^{14}CO_2$ (van Rysselberge & Jeener, 1955) suggests that the small anomalous particles in sap are more likely to be intermediaries in synthesis than breakdown products. The infectivity and nucleic acid content reported for aggregated preparations of these particles has varied (Bawden & Pirie, 1956*a*) perhaps because they were aggregated by different methods and after the sap had been exposed to different conditions, in which either TMV or other nucleic acid might have been degraded to different extents. The yield of infective, re-aggregated particles from alkali-treated TMV clearly depends on the conditions to which the preparations are exposed before they are aggregated.

The effects of alkali may also provide an analogy for what happens when leaves become infected. The amount of virus recoverable from inoculated leaves at first decreases and, as experiments with TMV labelled with ^{32}P show, P that was in the virus soon appears in other forms. Whether this derives from virus which is causing infections, or from excess inoculum, is impossible to say, but that the virus particles may pass through a temporary phase of susceptibility to pancreatic ribonuclease is suggested by the ability of this enzyme to inhibit infections when leaves are rubbed with solutions of it within 2 hr. of inoculation with the virus, but not afterwards. Similarly, photoreactivation experiments with potato virus X (Bawden & Kleczkowski, 1955) suggest that these particles undergo changes within 30 min. of being inoculated to leaves and that they are unstable in their changed state. All this combines to suggest that systems in host cells may act rather like alkali and disrupt virus particles to produce an infective component that is more labile than the particles characteristic of *in vitro* preparations.

The fact that infective material can often be separated from non-infective fragmented preparations of TMV is far from necessarily meaning that infective particles have been created by combining intrinsically non-infective components. It may mean no more than that an inhibitor of infection has been removed, or that infective fragments, which otherwise would have become non-infective before testing, have been stabilized *in vitro*. These possibilities are open to experimentation, and all our results that at first sight suggested the synthesis of infective particles became interpretable in other ways.

Removal of inhibitors or stabilization *in vitro* could also explain most claims to have synthesized infective virus from non-infective components, but it may be inadequate to explain the most recent results of Commoner *et al.* (1956). They used heated nucleic acid, which seems to exclude the possibility that material was present with the infective qualities we describe, and yet they report a greater infectivity in their 'reconstituted' particles than any reported by other workers. If this is confirmed, and their protein preparations are free from nucleic acid, then a phenomenon operationally indistinguishable from creating infectivity seems to have been achieved. Whether it has been created, however, or whether an exceptionally labile component has been stabilized against the hazards encountered during or after inoculation, will still remain in doubt. We got no infections with heated nucleic acid and have failed to increase the infectivity of alkali-treated products of TMV by aggregating them with heated nucleic acid, but we do not have the absorbent materials with which Commoner *et al.* (1956) removed nucleic acid from their alkali-treated preparations and so cannot use their experimental methods.

Study of the fission products of TMV suggests that immunological specificity is primarily an attribute of the protein and infectivity of the nucleic acid. There is no *a priori* reason why nucleic acid alone should not carry the infectivity, and the inactivation by ribonucleases points to it being an essential constituent of the infective unit. Indeed, the simplest interpretation of current results is that nucleic acid is the whole of this unit, and there is no compelling reason to look for any other component. There would be some reason if it should be established unequivocally that combination of non-infective nucleic acid with another substance gave an infective product, but even then it could happen because the product contained the right amount of nucleic acid arranged in the correct way. Before we agree with the conclusion that nucleic acid alone is the vehicle of infectivity, two points need putting. First, the infectivity of nucleic acid preparations is so slight that it could be accounted for by a 0·1 % contamination with some other unit possessing the infectivity of intact TMV, and to establish nucleic acid positively as the bearer would need demonstrating that the nucleic acid was 99·9 % pure, which is patently impossible. Even if the range of possible contaminants is narrowed by postulating a single substance, such as a protein, it would remain difficult to demonstrate convincingly that this was not present to the extent of 0·1 %. Secondly, our results with urea-treated TMV show that the infectivity of nucleic acid preparations depends on how they are made, and that substances harmless to phenol-made products yield non-infective products. There are many possible explanations for this, but one worth some consideration is that phenol-made products contain something other than nucleic acid and that this is lost when the virus is disrupted by urea.

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REFERENCES

Bawden, F. C. (1954). Inhibitors and plant viruses. *Advanc. Virus Res.* **2**, 31.

Bawden, F. C. (1956). Reversible, host-induced changes in a strain of tobacco mosaic virus. *Nature, Lond.* **177**, 302.

Bawden, F. C. & Kleczkowski, A. (1955). Studies on the ability of light to counteract the inactivating action of ultraviolet radiation on plant viruses. *J. gen. Microbiol.* **13**, 370.

Bawden, F. C. & Pirie, N. W. (1937). The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc. roy. Soc.* B, **123**, 274.

Bawden, F. C. & Pirie, N. W. (1940*a*). The effects of alkali and some simple organic substances on three plant viruses. *Biochem. J.* **34**, 1278.

Bawden, F. C. & Pirie, N. W. (1940*b*). The inactivation of some plant viruses by urea. *Biochem. J.* **34**, 1258.

Bawden, F. C. & Pirie, N. W. (1945). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.

Bawden, F. C. & Pirie, N. W. (1956*a*). Observations on the anomalous proteins occurring in extracts from plants infected with strains of tobacco mosaic virus. *J. gen. Microbiol.* **14**, 460.

Bawden, F. C. & Pirie, N. W. (1956*b*). In *The Nature of Viruses*, p. 37, p. 82 *et passim*. Ciba Foundation Symp. Ed. G. E. W. Wolstenholme & E. C. P. Millar. London: J. and A. Churchill Ltd.

Bawden, F. C., Pirie, N. W., Bernal, J. D., & Fankuchen, I. (1936). Liquid crystalline substances from virus-infected plants. *Nature, Lond.* **138**, 1051.

Best, R. J. (1936). The relationship between the activity of tobacco mosaic virus suspensions and hydrion concentration over the pH range 5 to 10. *Austr. J. exp. Biol. med. Sci.* **14**, 323.

Commoner, B., Lippincott, J. A., Shearer, G. B., Richman, E. E. & Wu, J. H. (1956). Reconstitution of tobacco mosaic virus components. *Nature, Lond.* **178**, 767.

Commoner, B., Newmark, P. & Rodenberg, S. D. (1952). An electrophoretic analysis of tobacco mosaic virus biosynthesis. *Arch. Biochem. Biophys.* **37**, 15.

Crook, E. M. & Sheffield, F. M. L. (1946). Electron-microscopy of viruses: 1. State of aggregation of tobacco mosaic virus. *Brit. J. exp. Path.* **27**, 328.

Fraenkel-Conrat, H. (1956). The role of the nucleic acid in the reconstitution of active tobacco mosaic virus. *J. Amer. chem. Soc.* **78**, 883.

Fraenkel-Conrat, H. & Williams, R. C. (1955). Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc. nat. Acad. Sci., Wash.* **41**, 690.

Gierer, A. & Schramm, G. (1956). Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature, Lond.* **177**, 702.

Hart, R. G. & Smith, J. D. (1956). Interactions of ribonucleotide polymers with tobacco mosaic virus protein to form virus-like particles. *Nature, Lond.* **178**, 739.

Holden, M. & Pirie, N. W. (1955*a*). The partial purification of leaf ribonuclease. *Biochem. J.* **60**, 39.

Holden, M. & Pirie, N. W. (1955*b*). A comparison of leaf and pancreatic ribonuclease. *Biochem. J.* **60**, 53.

Jeener, R. & Lemoine, P. (1953). Occurrence in plants infected with tobacco mosaic virus of a crystallizable antigen devoid of ribonucleic acid. *Nature, London.* **171**, 935.

Kleczkowski, A. (1946). Combination between different proteins and between proteins and yeast nucleic acid. *Biochem. J.* **40**, 677.

Kleczkowski, A. (1957). A preliminary study of tobacco mosaic virus by the gel diffusion precipitin tests. *J. gen. Microbiol.* **16**, 405.

Lippincott, J. A. & Commoner, B. (1956). Reactivation of tobacco mosaic virus infectivity in mixtures of virus protein and nucleic acid. *Biochim. biophys. Acta*, **19**, 198.

Loring, H. S. (1942). The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease. *J. gen. Physiol.* **25**, 497.

Pirie, N. W. (1954). The fission of tobacco mosaic virus and some other nucleoproteins by strontium nitrate. *Biochem. J.* **56**, 83.

Pirie, N. W. (1956). Some components of tobacco mosaic virus preparations made in different ways. *Biochem. J.* **63**, 316.

Pirie, N. W. (1957). Macromolecular nucleoproteins from healthy tobacco leaves. *Biochimia.* **22**, 140.

RYSSELBERGE, C. VAN & JEENER, R. (1955). The role of the soluble antigens in the multiplication of the tobacco mosaic virus. *Biochim. biophys. Acta*, **17**, 158.

SCHRAMM, G. (1943). Über die Spaltung des Tabakmosaikvirus in niedermolekulare Proteine und die Rückbildung hochmolekularen Proteins aus den Spaltstücken. *Naturwissenschaften*, **31**, 94.

SCHRAMM, G., SCHUMACHER, G. & ZILLIG, W. (1955). An infectious nucleoprotein from tobacco mosaic virus. *Nature, Lond.* **175**, 549.

SIEGEL, A., WILDMAN, S. G. & GINOZA, W. (1956). Sensitivity to ultraviolet light of infectious tobacco mosaic virus nucleic acid. *Nature, Lond.* **178**, 1117.

SREENIVASAYA, M. & PIRIE, N. W. (1938). The disintegration of tobacco mosaic virus preparations with sodium dodecyl sulphate. *Biochem. J.* **32**, 1707.

TAKAHASHI, W. N. & ISHII, M. (1952). The formation of rod-shaped particles resembling tobacco mosaic virus by polymerization of a protein from mosaic diseased tobacco leaves. *Phytopathology*, **42**, 690.

TAKAHASHI, W. N. & ISHII, M. (1953). A macromolecular protein associated with tobacco mosaic virus infection: its isolation and properties. *Amer. J. Bot.* **40**, 85.

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The Production of Polyenic Antibiotics by Soil Streptomycetes

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SUMMARY: Sixty Streptomyces isolates which produce polyene antibiotics were isolated from 25 soil samples collected in different parts of the world. Of these isolates 25 produced tetraenes, 23 produced pentaenes, 1 produced a hexaene, and 15 produced heptaenes. One isolate produced simultaneously a tetraene and a pentaene, another produced a tetraene and a heptaene, and a third produced a tetraene, a pentaene and a heptaene. The antibiotics showed activity against a wide range of fungi; the minimum inhibitory concentrations against *Saccharomyces cerevisiae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans*, *Fusarium culmorum* and *Trichophyton mentagrophytes* are given for 10 of them. The distribution of activity between culture filtrate and mycelium is given for 14 of the antibiotics.

Hazen & Brown (1951) described the isolation of an antifungal antibiotic (fungicidin) from a *Streptomyces* sp. It was active against a wide range of yeasts and other fungi, but not against bacteria. The ultraviolet (u.v.) absorption spectrum of the antibiotic in ethanol showed sharp maxima at 290, 305 and 320 mμ.; the frequency differences between these absorption peaks is characteristic of a series of conjugated double bonds, and the chromophoric grouping of the compound is of polyene type. Many other antibiotics of similar properties have been described in recent years in publications from the U.S.A. or Japan, and they may be grouped together on the basis of certain general properties:

(1) They inhibit the growth of a wide range of fungi, including yeasts, but are inactive against bacteria.

(2) They show a relatively high toxicity when injected into animals, but are much less toxic when given by mouth.

(3) They are of low solubility in water but dissolve more readily in aqueous solutions of the lower alcohols, and are easily soluble in aqueous pyridine.

(4) They exhibit characteristic ultraviolet absorption spectra typical of polyenic chromophores.

These properties are fairly general for the whole series of compounds, although there are some wide differences, particularly in their biological properties. Several workers have classified the polyenic antifungal antibiotics into a number of different groups according to the nature of their ultraviolet absorption spectra (Vining, Taber & Gregory, 1955; Utahara, Okami, Nakamura & Umezawa, 1954; Oroshnik, Vining, Mebane & Taber, 1955; Pledger & Lechevalier, 1956), and we have used a similar method of classification in a screening programme for antifungal antibiotics produced by various *Streptomyces* spp. The scheme we have used is shown in Table 1, together with the

Table 1. *The classification of polyene antibiotics according to their ultraviolet absorptions*

Amphotericins A and B: Vandeputte, Wachtel & Stiller (1956); antibiotic 1968: Oswald, Reedy & Randall (1956); antimycoin: Raubitschek, Acker & Waksman (1952); ascosin: Hickey, Corum, Hidy, Cohen, Nager & Kropp (1952); candicidin: Lechevalier, Acker, Corke, Haenseler & Waksman (1953); candidin: Taber, Vining & Waksman (1954); candimycin: Shibata, Honjo, Tokui & Nakazawa (1954); chromin: Wakaki, Hamada, Akanabe & Asahina (1953); eurocidin: Okami, Wahara, Nakamura & Umezawa (1954); filipin: Whitfield, Brock, Amman, Gottlieb & Carter (1955); flavacid: Takahashi (1953); fradicin: Swart, Romano & Waksman (1950); fungichromatin: Tytell, McCarthy, Fisher, Bolhofer & Charney (1955); fungichromin: Tytell *et al.* (1955); fungicidin: Hazen & Brown (1951); mediocidin: Okami *et al.* (1954); rimocidin: Davisson, Tanner, Finlay & Solomons (1951); trichomycin: Hosoya, Komatsu, Soeda & Sonoda (1952).

Group no.	Polyene type	Absorption maxima (mμ)	Published antibiotics
1	Tetraene	290, 305, 318	Fungicidin, antimycoin, rimocidin, chromin, amphotericin A
2A	Pentaene	318, 333, 351	Fungichromatin, eurocidin
2B	Pentaene	325, 340, 358	Fungichromin, filipin
3	Hexaene	340, 356, 378	Mediocidin, flavacid, fradicin
4	Heptaene	360, 378, 405	Ascosin, candicidin, candidin, trichomycin, candimycin, amphotericin, B, antibiotic 1968

recorded antibiotics placed in their respective classes. During 1952–3 we carried out a screening programme for antifungal antibiotics that might be used clinically and found a wide range of polyene antibiotics from microorganisms derived from soil samples collected in different parts of the world. The details of our methods and a selection of our results are given below.

METHODS

Pure cultures of the *Streptomyces* spp. were isolated from agar soil dilution plates. Four media were generally used for each soil—Bennett's, potato-peptone, yeast extract and paraffin agar. The compositions of these media were:

(1) Bennett's agar medium: NZ amine (enzymic casein digest from Sheffield Chemical Co. Inc., U.S.A.) 0·2 g., Difco beef extract 0·1 g., Difco yeast extract 0·1 g., glucose 1 g., agar 1·5 g., distilled water to 100 ml.; pH adjusted to 7·8 ± 0·1.

(2) Potato-peptone agar medium: aqueous extract from 20 g. of overcooked potatoes in 100 ml. of water 50 ml., peptone 0·5 g., dipotassium monohydrogen phosphate 0·2 g., sodium chloride 0·2 g., ferrous sulphate heptahydrate 0·001 g., agar 1·5 g., distilled water to 100 ml.; pH adjusted to 6·4 ± 0·1.

(3) Yeast extract agar medium: Difco yeast extract 1 g., Evans peptone 1 g., agar 1·5 g., distilled water to 100 ml.; pH adjusted to 7·7 ± 0·1.

(4) Paraffin agar medium: ammonium nitrate 0·4 g., liquid paraffin B.P. 0·1 g., dipotassium monohydrogen phosphate 0·6 g., potassium dihydrogen phosphate 0·2 g., zinc sulphate heptahydrate 0·0049 g., ferrous sulphate

heptahydrate 0·00054 g., manganous chloride tetrahydrate 0·0046 g., copper sulphate pentahydrate 0·00025 g., sodium borate 0·000094 g., ammonium molybdate 0·00002 g., agar 1·5 g., distilled water to 100 ml.; pH adjusted to 7·0 ± 0·1. The medium was kept hot to melt the agar and was vigorously stirred throughout the pouring of the plates.

The medium which was successful in producing the largest number of Streptomyces colonies varied with the soil population, but paraffin agar proved to be selective for *Streptomyces* spp. and sometimes permitted their isolation from soils containing a high content of spreading bacteria and fungi. The number of colonies isolated from any one soil varied from about 30 to 200/*g*. soil.

A primary screening for antifungal activity was carried out by an agar disc method. The Streptomyces isolates were grown, in small aluminium cups with loose fitting caps, on medium 1, 2 or 3 above. After 5 days of incubation at 28°, cork borer plugs of the grown cultures were transferred to test plates seeded with *Saccharomyces cerevisiae* and *Aspergillus niger*. The plates were incubated overnight at 37° and the Streptomyces which showed good inhibition zones were selected for investigation of their ability to produce antibiotics in submerged aerated culture. In this secondary screening, 2 ml. portions of a 48 hr. shake-flask culture in glycerol meat broth were used to inoculate duplicate flasks of 6 different media distributed in 40 ml. quantities in 250 ml. conical flasks. These flasks were incubated on a reciprocating shaker for 5 days at 28° and were sampled aseptically at daily intervals for assessment of their antibiotic content by a cup plate method using as test organism *Saccharomyces cerevisiae* NCTC 4614 in 2 % (w/v) glucose nutrient agar with 8 mm. diameter punched holes. When conditions had been found whereby a culture fluid gave an inhibition zone of more than 20 mm. overall diameter, six 250 ml. flasks containing 40 ml. medium were grown for extraction purposes. The six cultures were bulked and filtered without addition of a filter aid. The separated mycelium was extracted with 2 × 100 ml. 80 % (v/v) acetone in water, a stirring time of 15 min. being allowed for each extraction. The acetone was removed from the extract by concentration to small bulk (5 ml.) *in vacuo*; 20 ml. *n*-butanol was added to the aqueous extract and the water was removed by further concentration *in vacuo* to 5 ml. The antibiotic present in this extract was precipitated by the addition of 6 vol. diethyl ether and the resulting solid isolated by centrifugation, washed with ether to remove traces of butanol and dried *in vacuo* at 40° for 1 hr. The bulked culture filtrate was extracted with 2 × 100 ml. *n*-butanol, the combined butanol solutions were concentrated to 100 ml. *in vacuo*, and the solid was isolated as above. The solid preparations were examined in aqueous solution or in 40 % (v/v) pyridine in water for antibiotic activity, but ethanolic solutions were used for measurement of the ultraviolet absorption spectra in a Unicam model SP. 500 spectrophotometer. We found it satisfactory to take the absorption spectra at the butanolic concentrate stage, for the impure nature of the samples at this stage did not preclude recognition of the polyene entities, since sharp maxima were observed. It was necessary to dilute the solutions before

Table 2. *Distribution in soils of streptomycete isolates which produce polyene antibiotics*

Sample no.	Soil location	No. of polyene producing isolates	Type of polyene produced: Group 1	Group 2A	Group 2B	Group 3	Group 4
1	Argentine	3	2	3	.	.	1
2		1	.	.	.	.	1
3		1	1	.	.	.	.
4		2	2	.	.	.	1
5		2	.	.	.	.	2
6		1	.	.	1	.	.
7		4	1	3	.	.	.
8		2	.	2	.	.	.
9	Brazil	1	.	.	.	.	1
10	Burma	9	.	9	.	.	.
11	Ceylon	1	.	.	.	.	1
12		1	1	.	.	.	.
13	England	1	1	.	.	.	.
14	India	4	1	.	.	.	3
15	Iraq	3	1	.	.	.	2
16	Kenya	1	.	.	.	.	1
17	Malaya	1	.	1	.	.	.
18	Nigeria	1	.	.	1	.	.
19		1	.	.	1	.	.
20		2	2	.	.	.	.
21		13	13	.	.	.	.
22	Pakistan	2	.	.	.	.	2
23	Sierra Leone	1	.	1	.	.	.
24		1	.	.	.	1	.
25		1	.	1	.	.	.
Totals		60	25	20	3	1	15

Table 3. *Minimum inhibitory concentrations of different polyene-antibiotic preparations against 6 fungi*

Test organisms: *Saccharomyces* (*S.*) *cerevisiae, Aspergillus* (*A.*) *niger, Mucor* (*M.*) *racemosus, Candida* (*C.*) *albicans, Fusarium* (*F.*) *culmorum, Trichophyton* (*T.*) *mentagrophytes.*

Antibiotic no.	Polyene group	Estimated purity by ultraviolet absorption (%)	Test organism: *S. cerevisiae*	*A. niger*	*M. racemosus*	*C. albicans*	*F. culmorum*	*T. mentagrophytes*
			Minimum inhibitory concentration at 48 hr. (μg/ml.)					
A. 432	1	20–30	1·2	4·4	4·4	3·2	2·2	11·6
A. 228	2A	20–30	3·4	4·4	5·0	4·2	6·75	4·5
A. 688	2A	20–30	1·4	8·9	5·8	>3·25	8·9	9·75
A. 695	2A	20–30	2·2	20·0	2·2	2·2	6·6	20·0
A. 789	2A	20–30	1·5	4·4	2·9	6·6	5·3	12·0
A. 246	2B	80–90	0·66	0·66	4·4	2·2	2·2	4·4
A. 772	2B	20–30	2·96	4·44	10·0	4·44	2·96	4·44
A. 583	4	40–50	0·10	18·4	0·54	0·16	18·4	14·8
A. 570	4	20–30	0·48	16·0	5·3	1·43	16·0	16·0
A. 571	4	20–30	1·5	18·4	5·4	3·6	14·2	15·8

measurement; ethanol was used, as the absorption spectra did not vary greatly with solvent and the addition of ethanol gave clear solutions.

The antibiotic contents of the culture filtrates and mycelial extract samples were estimated by an agar cup-plate assay with *Saccharomyces cerevisiae* as the test organism. For the first extract of each antibiotic a sample of culture filtrate was used as an arbitrary assay standard. Usually enough solid was isolated from this extraction to enable all subsequent work on the antibiotic to be assayed in terms of the solid preparation in 40 % (v/v) pyridine in water. The minimum inhibitory concentrations against six selected fungi were determined by duplicate serial two-thirds or one-third dilutions in 2 % glucose broth at pH 6·0: 48 hr. broth cultures diluted 1/100 were used as inocula and dilution tubes were incubated at 28° for 48 hr. The test organisms used were *Saccharomyces cerevisiae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans*, *Fusarium culmorum* and *Trichophyton mentagrophytes*.

RESULTS

In 25 soil samples from various localities 60 polyene antibiotic-producing isolates were found. One isolate produced simultaneously antibiotics in groups 1 and 2A, another produced antibiotics in groups 1 and 4, and a third isolate produced antibiotics in groups 1, 2A and 4. Three isolates produced antibiotics which were not of a polyene nature. In two of these the antibiotic was identified as cycloheximide (Whiffen, Bohonos & Emerson, 1946) but the third one was not characterized. The locations of the soil samples and the polyene-producing isolates obtained therefrom are given in Table 2, together with the types of polyene they produce. A selection of some results for the minimal inhibitory concentrations of the antibiotics against 6 fungi and yeasts is given in Table 3.

The relative amounts of the antibiotics contained in the mycelia and culture filtrates of the organisms grown in different media were determined by plate assay against *Saccharomyces cerevisiae*. Whole cultures and culture filtrates were assayed directly, and the mycelia were extracted exhaustively with 80 % (v/v) acetone in water, the acetone being then removed *in vacuo* before dilution and addition to the plates. Some of the results are shown in Table 4.

Antibiotics of group 1 assayed as whole culture always gave an accurate assessment of the total antibiotic present, that is, all the antibiotic was available for assay purposes. This was not true of antibiotics in the other groups, where the figures obtained by whole culture assay were always lower than the amount of antibiotic activity recovered by extraction. In these cases part of the antibiotic is held in a bound form by the mycelium and is not available for assay purposes.

DISCUSSION

It seems from our results that there is a wide distribution throughout the world of streptomycetes which produce polyene antibiotics, though we can draw no conclusions about their abundance. The distribution of polyenes found by us differs markedly from that described by Pledger & Lechevalier (1956), but this is not surprising in view of the limited range of soils they examined. Further, they stated that their method of screening favoured the selection of *Streptomyces griseus* strains, a limitation that does not apply to our methods of isolation. We think it noteworthy that most antifungal antibiotics revealed by our screening programme are of a polyene nature. The ratio of polyene antibiotics to non-polyene types found was 20 to 1.

Table 4. *Distribution of activity of polyene antibiotics between culture filtrates and mycelia*

		Activity (%)	
Antibiotic no.	Polyene type	Culture filtrate	Mycelium
A. 284	1	50	50
A. 288	1	31	69
A. 387	1	0	100
A. 432	1	7	93
A. 862	1	67	33
A. 228	2A	55	45
A. 341	2A	78	22
A. 786	2A	57	43
A. 789	2A	24	76
A. 246	2B	40	60
A. 772	2B	47	53
A. 4	4	40	60
A. 571	4	7	93
A. 583	4	40	60

There does not appear to be any correlation of polyene type with geographical location, although the number of soil samples investigated is too small for drawing any firm conclusions. We consider any such relationship rather improbable in view of the general kind of distribution we have found. Further, the fact that one isolate can produce more than one type of polyene would appear inconsistent with any such relationship, even though all the isolates which produced more than one polyene originated in the Argentine.

The results obtained with the limited number of test organisms used show that there are small differences between antibiotics in the same group and that members of groups 1, 2A and 2B cannot be distinguished by the test organisms used. Group 4 antibiotics differ markedly from those of groups 1, 2A and 2B by their relative inactivity against three of the four filamentous fungi and their high activity against yeasts. In the *Saccharomyces cerevisiae* plate assay it was noted that antibiotics of groups 1, 2A and 2B gave satisfactory dose-response curves (2·5 mm. diam. for doubling dilutions) and sharp inhibition zones. Antibiotics of group 4 and the one group 3 antibiotic gave poor dose-response curves (about 1·5 mm. for doubling dilutions) and zones with diffuse edges. A point of distinction between antibiotics of group 1 and those of the other groups was in the reliability of the whole culture assay values. Whereas the group 1 antibiotics always gave reliable results when the cultures were assayed on plates, the members of the other groups generally gave by this assay figures well below the true antibiotic contents of the cultures, as determined by extractions. However, there was no relationship between the type of polyene and the distribution of activity between culture filtrate and mycelium. The widely differing values for the proportions of antibiotic in culture filtrates and mycelia, and in particular the high filtrate contents, are somewhat surprising, since the pure antibiotics have low solubilities in water. It would have appeared reasonable to expect accumulations of the antibiotics in the mycelia, but this did not occur, and there must be active solubilizing agents present in culture media used.

REFERENCES

DAVISSON, J. W., TANNER, JR., F. W., FINLAY, A. C. & SOLOMONS, I. A. (1951). Rimocidin, a new antibiotic. *Antibiot. & Chemother.* **1**, 289.

HAZEN, E. L. & BROWN, R. (1951). Fungicidin, an antibiotic produced by a soil Actinomycete. *Proc. Soc. exp. Biol., N.Y.* **76**, 93.

HICKEY, R. J., CORUM, C. J., HIDY, P. H., COHEN, I. R., NAGER, U. F. B. & KROPP, E. (1952). Ascosin, an antifungal antibiotic produced by a Streptomycete. *Antibiot. & Chemother.* **2**, 472.

HOSOYA, S., KOMATSU, N., SOEDA, M. & SONODA, Y. (1952). Trichomycin, a new antibiotic produced by *Streptomyces hachijoensis* with trichomonadicidal and antifungal activity. *Jap. J. exp. Med.* **22**, 405.

LECHEVALIER, H., ACKER, R. F., CORKE, C. T., HAENSELER, C. M. & WAKSMAN, S. A. (1953). Candicidin, a new antifungal antibiotic. *Mycologia*, **45**, 155.

OKAMI, Y., WAHARA, R., NAKAMURA, S. & UMEZAWA, H. (1954). Studies on antibiotic Actinomycetes. IX. *J. Antibiot. Japan*, A, **7**, 98.

OROSHNIK, W., VINING, L. C., MEBANE, A. D. & TABER, W. A. (1955). Polyene antibiotics. *Science*, **121**, 147.

OSWALD, E. J., REEDY, R. J. & RANDALL, W. A. (1956). An antifungal agent, 1968, produced by a new *Streptomyces* species. *Antibiotics Annual*, 1955–6, p. 236. New York Medical Encyclopaedia Inc.

PLEDGER, R. A. & LECHEVALIER, H. (1956). Survey of the production of polyenic substances by soil Streptomycetes. *Antibiotics Annual*, 1955–6, p. 249. New York Medical Encyclopaedia Inc.

RAUBITSCHEK, F., ACKER, R. F. & WAKSMAN, S. A. (1952). Production of an antifungal agent of the fungicidin type by *Streptomyces aureus*. *Antibiot. & Chemother.* **2**, 179.

SHIBATA, M., HONJO, M., TOKUI, Y. & NAKAZAWA, K. (1954). On a new antifungal and antiyeast substance, Candimycin, produced by a Streptomyces. *J. Antibiot. Japan*, B, **7**, 168.

SWART, E. A., ROMANO, A. H. & WAKSMAN, S. A. (1950). Fradicin, an antifungal agent produced by *Streptomyces fradiae*. *Proc. Soc. exp. Biol., N.Y.* **73**, 376.

TABER, W. A., VINING, L. C. & WAKSMAN, S. A. (1954). Candidin, a new antifungal antibiotic produced by *Streptomyces viridoflavus*. *Antibiot. & Chemother.* **4**, 455.

TAKAHASHI, I. (1953). A new antifungal substance, flavacid. Studies on the antibiotic substances from Actinomycetes, XXVII. *J. Antibiot. Japan*, A, **6**, 117.

TYTELL, A. A., MCCARTHY, F. J., FISHER, W. P., BOLHOFER, W. A. & CHARNEY, J. (1955). Fungichromin and Fungichromatin: new polyene antifungal agents. *Antibiotics Annual* 1954–5. p. 716. New York Medical Encyclopaedia Inc.

UTAHARA, R., OKAMI, Y., NAKAMURA, S. & UMEZAWA, H. (1954). On a new antifungal substance, Mediocidin, and other antifungal substances of Streptomyces with three characteristic absorption maxima. *J. Antibiot. Japan*, A, **8**, 120.

VANDEPUTTE, J., WACHTEL, J. L. & STILLER, E. T. (1956). Amphotericins A and B, antifungal antibiotics produced by a Streptomycete. II. The isolation and properties of the crystalline amphotericins. *Antibiotics Annual* 1955–6, p. 587. New York Medical Encyclopaedia Inc.

Vining, L. C., Taber, W. A. & Gregory, F. J. (1955). The candidin-candicidin group of antifungal antibiotics. *Antibiotics Annual* 1954–5, p. 980. New York Medical Encyclopaedia Inc.

Wakaki, S., Hamada, K., Akanabe, S. & Asahina, T. (1953). Studies on the antifungal antibiotic from Streptomyces. IV. On the physico-chemical properties of chromin. *J. Antibiot. Japan*, A, **6**, 145.

Whiffen, A. J., Bohonos, N. & Emerson, R. J. (1946). The production of an antifungal antibiotic by *Streptomyces griseus*. *J. Bact.* **52**, 610.

Whitfield, G. B., Brock, T. D., Amman, A., Gottlieb, D. & Carter, H. E. (1955). Filipin, an antifungal antibiotic. Isolation and properties. *J. Amer. chem. Soc.* **77**, 4799.

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A New Genus of the Actinomycetales: *Waksmania* gen.nov.

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SUMMARY: A single mesophilic species of a new genus belonging to the family Streptomycetaceae of the order Actinomycetales is described and has been named *Waksmania* (*W. rosea*, type species). It produces a filamentous growth which is differentiated into vegetative (primary) mycelium and aerial (secondary) mycelium. Hyphae are 1·5 μ. or less in diameter. The new genus is characterized by the production of pairs of spores which are formed either: (1) at the tip of sporophores which branch from an aerially borne hypha; or (2) directly on an aerial hypha. Spores are not formed on the vegetative mycelium.

The micro-organisms commonly known as actinomycetes are thought by many to represent a group which is intermediate between the bacteria and the fungi. According to the most widely accepted systematic classification of these organisms, they are grouped under the order Actinomycetales (*Bergey's Manual of Determinative Bacteriology*, 1948). The organisms of the order Actinomycetales are described as those which form elongated cells which have a definite tendency to branch. Hyphae are 0·3–1·5 μ., usually about 1·0 μ. in diameter. Special 'fragmentation' spores, oidiospores, and conidia are formed by some genera. Briefly, the order is divided as follows (after Waksman & Henrici, 1943):

Mycelium rudimentary or absent, no spores formed.

Family I. Mycobacteriaceae

(1) Sole genus: *Mycobacterium*

True mycelium produced.

A. Vegetative mycelium divides by segmentation into bacillary or coccoid elements.

Family II. Actinomycetaceae

(1) Anaerobic: *Actinomyces*
(2) Aerobic: *Nocardia*

B. Vegetative mycelium normally undivided.

Family III. Streptomycetaceae

(1) Conidia produced in aerial hyphae in chains: *Streptomyces*
(2) Conidia produced terminally and singly on short conidiophores: *Micromonospora*

Couch (1949, 1950, 1954, 1955) isolated some previously undescribed actinomycetes which form sporangia. In some strains, motile spores were enclosed in the sporangia (*Actinoplanes*); in others, non-motile spores were present (*Streptosporangium*). Couch proposed that these new genera be placed in a new family, Actinosporangiaceae. Waksman & Corke (1953), in describing a new species of a thermophilic actinomycete, proposed the re-instatement of the genus *Thermoactinomyces* Tsiklinsky. Cultures of *Thermoactinomyces* are differentiated from the organisms of the genus *Micromonospora*, with which they had formerly been classified, by their ability to produce a true aerial mycelium, a characteristic which is lacking or appears in rudimentary form in *Micromonospora* spp. *Thermoactinomyces* spores are borne singly on sporophores on the vegetative and aerial mycelium. This genus would be classified under the family Streptomycetaceae.

The present paper reports the isolation of a new type of actinomycete which in some respects resembles *Streptomyces* and in others *Micromonospora* and *Thermoactinomyces*. This hitherto undescribed organism produces vegetative and aerial mycelium like *Streptomyces* and *Thermoactinomyces*. However, spores are formed in pairs, both directly on the aerial mycelium, and at the tip of sporophores which branch monopodially from a main aerial hypha. The name proposed for this new genus is *Waksmania*. The type species is *W. rosea*.

METHODS

Isolation

While examining a number of soils by using a slight modification of Couch's chytrid-isolating method (1939), a pink mycelial mass growing above the surface of the water on a particle of soil was observed. Following isolation to pure culture, this organism was found to have certain novel and previously undescribed characteristics.

Couch's method, as used here, may be described as follows: a scant teaspoonful of soil is placed in a sterile Petri dish and the dish flooded with sterile tap water. When the particles of soil have settled, several blades of grass, previously boiled for 15 min., are placed on, or just beneath the surface of, the water, and the plate is incubated at 28° for 1–5 weeks. Organisms growing on the grass and soil particles are observed and transferred with a sterile inoculating needle to a sterile agar medium, with the aid of a low-power objective.

Media for characterization

All media other than those described here are listed in Waksman's book on actinomycetes (1950). All complex organic constituents were products of Difco Laboratories (Detroit 1, Michigan, U.S.A.), except where otherwise noted. The soluble starch was a product of Merck and Co. Inc. (Rahway, New Jersey, U.S.A.).

Rice extract agar. Brown rice (River Brand Mills, New York, New York, U.S.A.) 200·0 g.; tap water, 1 l. agar, 15·0 g. Autoclave rice and water at 15 lb./sq. in. for 15 min. Add 750 ml. hot water while rice is still hot, stir for

1 min., decant and filter through cotton. Add agar. Do not adjust pH value.

Pablum extract agar. Pablum Mixed Cereal (Pablum Products, Div. of Mead, Johnson and Co., Evansville 21, Indiana, U.S.A.), 60·0 g.; tap water, 1 l.; agar, 15·0 g. Place dry cereal in four fine cheesecloth bags and tie with strings. Dip bags up and down in boiling water for 2–3 min. Extract will be brownish and slightly viscous. Drain excess moisture from bags and discard. Add agar to extract, bring up to 1 l. Do not adjust pH value.

Oatmeal agar. Rolled oats, 70·0 g.; tap water, 1 l.; agar, 15·0 g. Boil oats in water for 5 min., filter through fine cheesecloth, bring volume up to 1 l. while hot. Do not adjust pH value.

Gelatin. 12 % (w/v) in water.

Litmus milk. 105 g. prepared powder (100 g. skim milk; 5·0 g. litmus) in 1 l. distilled water.

Potato agar. Prepared pills. 1 pill for 10 ml. medium. (Ben Venu Laboratory Inc., Bedford, Ohio, U.S.A.)

Nitrate broth. Peptone, 5·0 g.; beef extract, 3·0 g.; KNO_3, 1·0 g.; distilled water, 1 l.

Carbon-free medium for testing cellulose utilization. K_2HPO_4, 1·0 g.; $NaNO_3$, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water to 1 litre, pH value adjusted to 7·5. Strips of filter-paper are added to the tubes before sterilization.

All media were sterilized for 15 min. at 15 lb./sq.in.

Tests for antibiotic properties

All the methods used were as described by Waksman (1950).

Stains

All staining procedures and solutions will be found in the *Manual of Methods for Pure Culture Study of Bacteria* (1951). *Methylene blue* 2 % aqueous solution (w/v); *Acid-fast stain* Ziehl-Neelsen method, slides being decolorized for 1, 5, and 10 sec.; *Gram method,* Hucker's modification; *Safranin* as given in Hucker's modification of the Gram method; *Crystal violet,* Hucker's modification.

Photographs

Photographs were taken through a trinocular American Optical 'Microstar' microscope with a 35 mm. Kodak photomicrography attachment. Microfile film (Eastman Kodak, Rochester, New York, U.S.A.) was developed in Kodak Mikrodol developer. Positives were made on Varigram paper (E. I. Du Pont de Nemours and Co. Inc., Wilmington 98, Delaware, U.S.A.), using appropriate filters. Electron photomicrographs were taken through an RCA EMU-1 electron microscope. Collodion films mounted on a grid were touched to the surface of a heavily sporulating agar culture, portions of the aerial mycelium becoming attached to the collodion membrane. Shadowing, when done, was with germanium at an angle of 26°.

RESULTS

Waksmania *Lechevalier et Lechevalier, gen.nov.*

Fine mycelium (about 1μ. in diameter) which is differentiated into (1) primary (vegetative) mycelium which grows into, and forms a compact layer on top of, agar media, and (2) secondary (aerial) mycelium which arises from the primary mycelium but grows into the air, away from the agar surface. The primary mycelium does not bear spores of any type; the secondary mycelium bears spores which are formed in longitudinal pairs. The spores are produced either directly on the aerial hyphae or on sporophores which branch from the aerial hyphae.

Type species, Waksmania rosea, *Lechevalier et Lechevalier, sp.nov.*

Morphology

Vegetative mycelium. 0·3–1·2μ. in diameter. Filaments long, branching, penetrating the agar medium and forming compact, tough colonies like those of *Streptomyces*. Hyphae do not segment, even in old cultures.

Aerial mycelium. 0·8–1·5μ., usually about 1·0μ., in diameter. Older hyphae tend to shrink in diameter. Some branching. Length of hyphae usually does not exceed 150μ. Hyphae are not erect, but lie along the surface of the medium, or at less than right angles to it. Occasionally, an aerial hypha will arise from the agar, grow along the surface for some time, bear spores, and then the growing tip will dip again under the surface. 'Fairy rings', or alternating areas of aerial mycelium with zones having none, common to other members of the Streptomycetaceae, occur on some media. 'Coremia-like' aggregations of aerial hyphae are formed on a variety of substrates.

Sporophores. Small branchlets are formed monopodially in respect to the main axis of the aerial sporogenous hyphae. Spores are borne at the tip of these branchlets or sporophores. Sporophore diameter varies from 0·3 to 2·0μ. This wider dimension represents that of the base of a sporophore such as illustrated in Fig. 1B. Length varies from almost negligible to 6·0μ., and is usually 1·0μ. or less. The sporophore does not appear to be cut off by a cross-wall from the main hypha (Pl. 1, figs. 8–10), and does not differ from it in appearance, except in diameter. Electron photographs also seem to reveal the presence of a cup or ring at the base of the spores borne on sporophores (Pl. 1, fig. 6). This detail is not apparent when the organism is viewed through the light microscope and may be an artifact.

Spores. Spherical to subglobose, 1·5–2·0μ., usually about 1·7–1·8μ. Spores are borne terminally on sporophores (Fig. 1C), as well as at the tips of the main sporogenous hyphae and branches (Fig. 2G). They are also borne directly on the sporogenous hyphae (Fig. 1A). Spores are formed in pairs, and when mature, are very easily detached from the sporophore and each other. Spores are sometimes borne eccentrically at the tip of the sporophore (Fig. 1D). None is formed on the vegetative hyphae, but occasionally the fall of an aerial filament into the agar will give rise to the impression that the spores are formed under the agar surface. Spore surface is smooth (Pl. 1, fig. 7).

Spore formation. A tiny bud on an aerial hypha grows to form a small branchlet (Pl. 1, fig. 8), which then appears to develop two 'septa'. The protoplasm of the portion of the branchlet destined to become spores becomes more re-fractile to light than the portion destined to become the sporophore. Stained preparations (Pl. 1, figs. 3–5) seem to indicate the presence of septa between spores; however, electron microphotographs do not confirm the presence of any cell wall (Pl. 1, figs. 7, 8). Both spores swell, not always simultaneously, and become more or less round (Fig. 2). On inoculation into

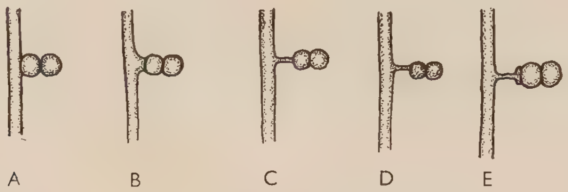

Fig. 1. *Waksmania rosea.* Spores borne: A, directly on the hypha; B, on a short sporophore; C, on a long sporophore; D, eccentrically on the sporophore; E, possible cup-shaped structure of the sporophore as suggested by electron microphotography.

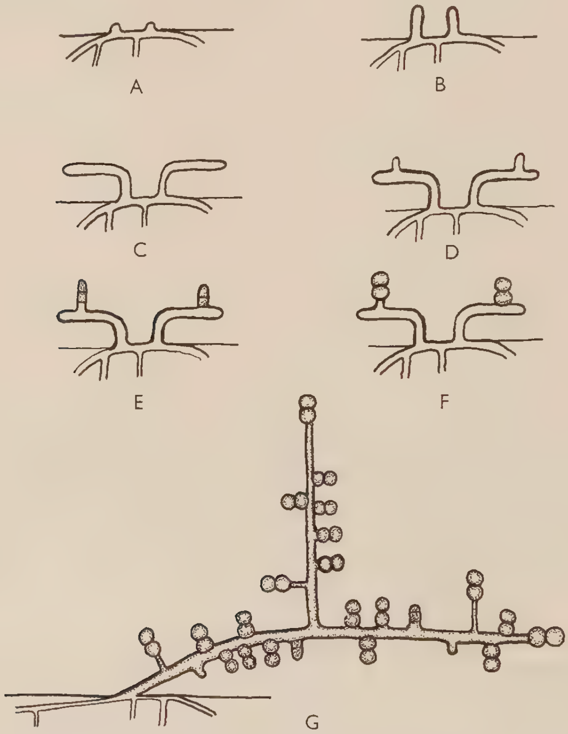

Fig. 2. *Waksmania rosea.* Schematic representation of the mode of formation of the aerial mycelium and spores. A, B, C, formation of aerial mycelium; D, E, F, formation of spores, one sessile and one with sporophore. At E note the condensation of the protoplasm of the future spore; G, schematic diagram of typical aerial hypha.

a fresh medium the spores germinate, producing one to three germ tubes. A very large number of spores never germinate.

Appearance on various media

All cultures were incubated at 28°. *Waksmania rosea* does not form a soluble pigment on any medium tested.

Yeast glucose agar. White-tan glistening growth becoming dark brown and convoluted. Aerial mycelium is not formed.

Glucose asparagine agar. Growth meagre, colourless. Aerial mycelium is not formed.

Czapek's agar. Thin yellowish white growth. Scant aerial mycelium. Some malformed spores formed.

Rice extract agar. Bright orange pink vegetative mycelium. White aerial mycelium with light pink spores borne profusely. Strong earthy odour.

Pablum extract agar. Shiny bright pink-orange to brown-orange vegetative mycelium becoming covered with white aerial mycelium. Pink spores borne in profusion. Earthy odour.

Oatmeal agar. Orange-pink vegetative growth. White aerial mycelium, light pink spores. Earthy odour.

Potato plug. Dark reddish brown growth. Very slight trace of white aerial mycelium.

Litmus milk. Completely peptonized after 1 month. No coagulation, no change in pH value. White fluffy growth on the bottom of the tube. Orange-pink surface colonies attached to sides of test tube.

Gelatin. Slightly attacked. White fluffy growth on the bottom of tube. No surface growth.

Cellulose utilization. Attacks cellulose to a very limited degree.

Nitrate broth. No nitrite after incubation for 3–5 weeks.

Starch utilization. (1) Starch agar A: no growth. (2) Starch agar B: good growth. No diastatic activity after 3 weeks. (3) Potato agar: moderate diastatic activity. Tests for starch utilization were made with a Lugol solution.

Staining properties

Waksmania rosea is not acid-fast and is Gram-positive. It stains well with crystal violet and safranin. Preparations stained with methylene blue show: (*a*) some developing spores which stain entirely blue with darker 'septa' (Pl. 1, fig. 5); (*b*) many spores unstained except for dark blue cross-walls cutting off the spores; (*c*) many mature spores which do not stain at all. The hyphae and sporophores do not take this stain strongly.

Growth temperature

The organism is a mesophil, growing well at 25–35°. It produces only a very sparse growth at 40°, and does not grow at all at 55°.

Antibiotic properties

No activity was found by cross-streak tests or in a variety of culture media incubated at 28° on a rotary shaking machine and tested by the streak dilution method. The following test organisms were used: *Candida albicans, Aspergillus niger, Saccharomyces cerevisiae, Rhizopus nigricans, Staphylococcus aureus, Sarcina lutea, Micrococcus lysodeikticus Escherichia coli.*

Source

Waksmania rosea was isolated from a fallow garden soil located in Martinsville, New Jersey, U.S.A. One strain of *W. rosea* is deposited in the Collection of the Institute of Microbiology of Rutgers University and bears the number 3748.

DISCUSSION

The new genus *Waksmania* should be included in the Streptomycetaceae. Its similarity to the other three members of the family may be summarized as follows:

(i) *Streptomyces* and *Waksmania* both produce: (*a*) aerial hyphae; (*b*) spores on the aerial hyphae only; (*c*) some spores directly on the sporogenous hyphae; (*d*) compact, leathery colonies that adhere to the agar; (*e*) non-wetting spores; (*f*) an earthy odour.

(ii) *Micromonospora* and *Waksmania* both form spores at the tip of sporophores. On some media *W. rosea* forms insoluble orange-pink pigments typical of many *Micromonospora* spp. and on many media on which it does not form aerial mycelium, its macroscopic aspect resembles this genus.

(iii) *Thermoactinomyces* and *Waksmania* both produce: (*a*) aerial mycelium; (*b*) some spores on sporophores; (*c*) an earthy odour; (*d*) non-wetting spores.

Although in certain *Streptomyces* spp. conidia are borne on sporophore-like branchlets, these sporophores are usually of the same diameter as the spores they bear, or occasionally when they are slightly smaller in diameter than the spores, this difference is about 0·1–0·2μ. In *Waksmania rosea*, the difference between the diameter of the sporophores and the spores is usually greater than 1·0μ. This more closely approaches the difference in diameter between the spore and sporophore of *Micromonospora* spp.

As a consequence, the following revision of the existing key to the family Streptomycetaceae is proposed:

I. Aerial (secondary) mycelium formed.

Spores formed singly (not in chains): *Thermoactinomyces* Tsiklinsky
Spores formed in pairs: *Waksmania* gen.nov.
Spores formed in chains: *Streptomyces* Waksman & Henrici

II. Aerial (secondary) mycelium not formed.

Spores formed singly (not in chains): *Micromonospora* Ørskov

The new genus *Waksmania* underlines and confirms the morphological relationship of *Streptomyces* to *Micromonospora* and *Thermoactinomyces*. Further, it represents a link between *Streptomyces* and *Thermoactinomycetes*. Physiologically, *Waksmania rosea* appears to be a relatively inactive member of the Streptomycetaceae. Furthermore, the limited number of media on

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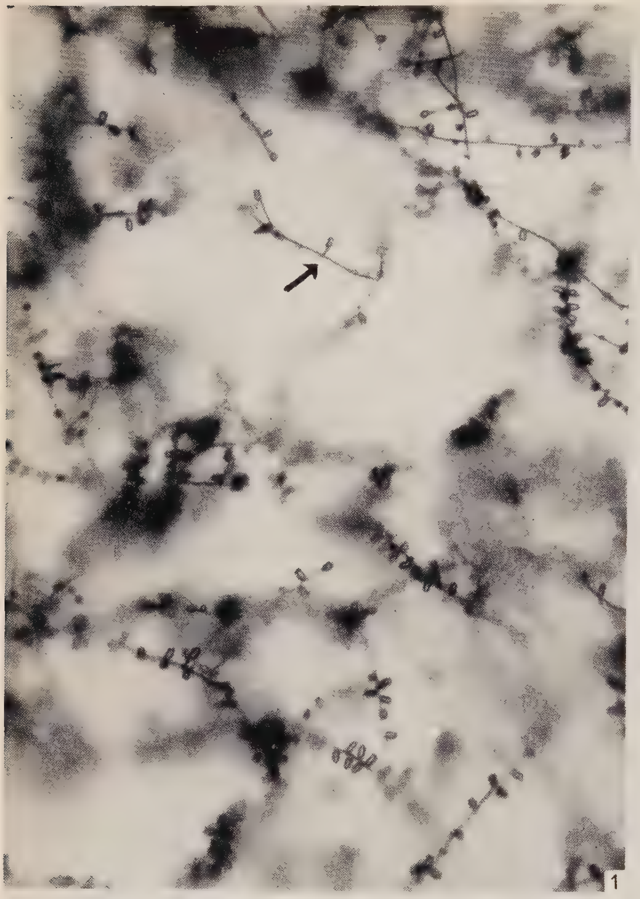
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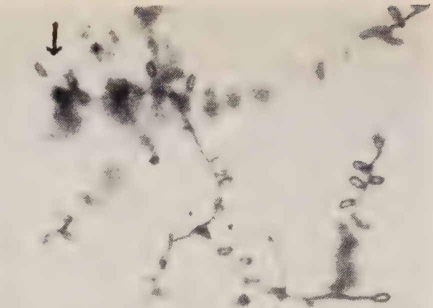
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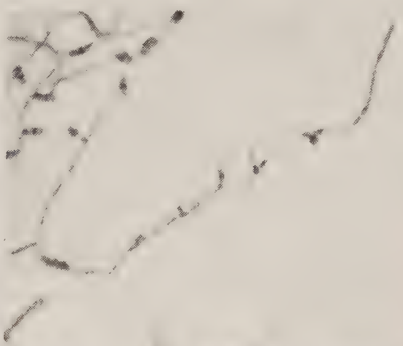
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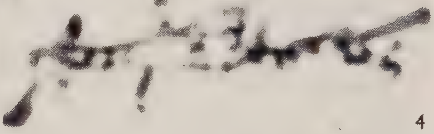
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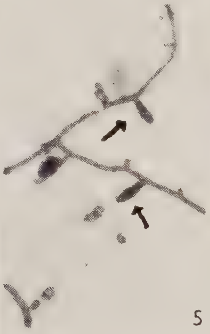
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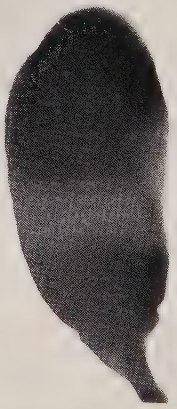
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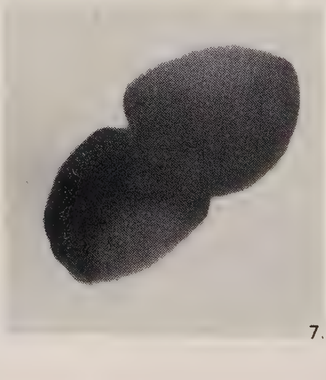
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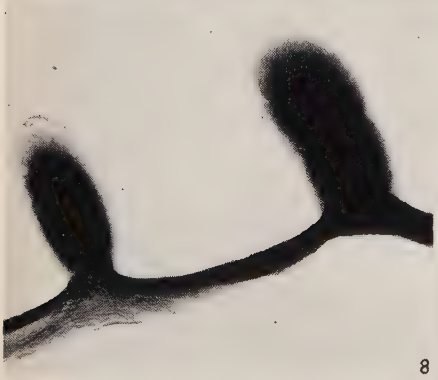
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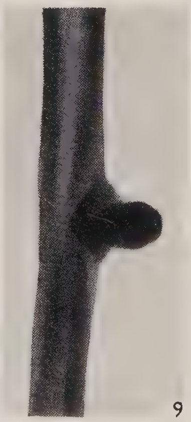
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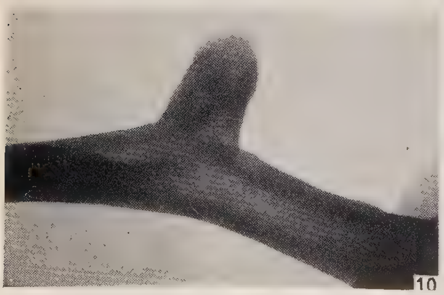
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M. P. Lechevalier and H. Lechevalier—A new actinomycete genus. Plate 1

(*Facing p.* 111)

which this organism will form spores indicates that its nutritional requirements are rather unusual. It remains to be seen whether even more radical departures from conventional methods of actinomycete isolation than those used here, will bring to light more new members of this group of important soil inhabitants.

The authors wish to acknowledge the invaluable assistance and advice of Dr Ruth E. Gordon of the Institute of Microbiology. To Miss Pauline Holbert, also of the Institute, we wish to express our sincere appreciation for taking and printing the electron micrographs. We are also greatly indebted to Dr Selman A. Waksman, Director of the Institute, for his authoritative advice on this paper.

REFERENCES

Bergey's Manual of Determinative Bacteriology (1948). 6th edition, edited by Breed, R. S., Murray, E. G. C., & Hitchens, A. P. Baltimore: Williams and Wilkins Co.

Couch, J. N. (1939). Technic for collection, isolation, and culture of chytrids. *J. Elisha Mitchell sci. Soc.* **55**, 208.

Couch, J. N. (1949). A group of organisms related to *Actinomyces*. *J. Elisha Mitchell sci. Soc.* **65**, 315.

Couch, J. N. (1950). *Actinoplanes*, a new genus of the *Actinomycetales*. *J. Elisha Mitchell sci. Soc.* **66**, 87.

Couch, J. N. (1954). The genus *Actinoplanes* and its relatives. *Trans. N.Y. Acad. Sci.* **16**, 315.

Couch, J. N. (1955). A new genus and family of the *Actinomycetales*, with a revision of the genus *Actinoplanes*. *J. Elisha Mitchell sci. Soc.* **71**, 148.

Manual of Methods for Pure Culture Study of Bacteria. Leaflet IV (1951). 10th edition, edited by Conn, H. J. and collaborators. Geneva, New York: Biotech Publications.

Waksman, S. A. (1950). *The actinomycetes.* Waltham, Mass.: Chronica Botanica Co.

Waksman, S. A. & Corke, C. T. (1953). *Thermoactinomyces* Tsiklinsky, a genus of thermophilic actinomycetes. *J. Bact.* **66**, 377.

Waksman, S. A. & Henrici, A. T. (1943). The nomenclature and classification of the actinomycetes. *J. Bact.* **46**, 377.

EXPLANATION OF PLATE

Fig. 1. Aerial hyphae on culture plates viewed directly through dry objective. Arrows indicate sporophores. × 420.

Fig. 2. As in fig. 1. × 525.

Fig. 3. Aerial hyphae stained with methylene blue. × 840.

Fig. 4. As in fig. 3. × 2310.

Fig. 5. As in fig. 3. × 1260. Note deeply stained immature spores at arrow.

Fig. 6. Electron micrograph of a pair of developing spores. Note cup-shaped apex of the sporophore. × 16,800.

Fig. 7. Electron micrograph of detached mature spores. × 18,900.

Fig. 8. Electron micrograph of young spores. × 11,200.

Figs. 9 and 10. Electron micrographs of sporophores. × 26,600.

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After this paper was accepted for publication, a description of a thermophilic actinomycete, morphologically similar to *Waksmania rosea*, was published by A. Henssen in the *Arch. Mikrobiol.* (1957): 'Beiträge zur Morphologie und Systematik der Thermophilen Actinomyceten', **26**, 373.

Huybers, K. & Jennings, P. (1957). *J. gen. Microbiol.* **17**, 112–116

A Pure Preparation of the Staphylococcal Typing Bacteriophage 42D

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SUMMARY: The staphylococcal typing-phage 42D is propagated on bacteria which are lysogenic for the phage 1363, and the latter phage is present as a contaminant in preparations of phage 42D. This contamination can be avoided by propagating a pure clone of phage 42D on non-lysogenic organisms. Such non-lysogenic propagating strains can be isolated after the lysogenic propagating organisms have been irradiated heavily with ultraviolet radiation.

In testing the reactions of twenty-five staphylococcal phages Rountree (1949) found that many produced a small number of plaques on organisms other than those of homologous type. As most of these phages had been propagated on lysogenic bacteria, she suggested that the anomalous reactions might be due to contamination of the preparations with phage derived from the propagating organisms. Williams & Rippon (1952) in their description of the techniques used to phage-type *Staphylococcus aureus* pointed out that preparations of the staphylococcal typing-phages might be contaminated in this fashion, for it is known (Rountree, 1949) that sixteen of the typing-phage propagating strains are lysogenic. It has recently been shown that preparations of the typing-phages 79 (Rippon, 1956) and 47C (Rountree, 1956) are in fact contaminated in this way. The following experiments show that preparations of typing-phage 42D are similarly contaminated.

By using non-lysogenic organisms which had appeared spontaneously on subculture of the lysogenic propagating strain Rountree (1956) obtained a pure preparation of the phage 47C. In the following experiments, non-lysogenic organisms were similarly used to obtain a pure preparation of phage 42D. These non-lysogenic organisms were obtained by irradiating the lysogenic propagating strain with a heavy dose of ultraviolet radiation, a technique first used by Lederberg & Lederberg (1953) to obtain non-lysogenic strains of *Escherichia coli* K12.

METHODS

Phages and organisms. The typing phage 42D is propagated on *Staphylococcus aureus* 1363. This organism is known to be lysogenic (Williams Smith, 1948; Rountree, 1949) and the phage it produces, which has not previously been named, is referred to here as phage 1363. The various strains of *S. aureus* 1363 described in this paper are referred to as follows: (*a*) 1363 (1363): *S. aureus* 1363 carrying the prophage 1363; (*b*) 1363 (–): *S. aureus* 1363, non-lysogenic; (*c*) 1363 (42D): *S. aureus* 1363 carrying the prophage 42D.

This latter strain was isolated as a derivative of a doubly lysogenic strain 1363 (1363 . 42 D) in the course of other experiments.

Phage 42 D forms plaques on staphylococci 1363 (1363) and 1363 (–), and phage 1363 forms plaques on staphylococci 1363 (42 D) and 1363 (–). In each case the efficiency of plating on the lysogenic and non-lyosgenic strain is the same.

Media. Bacto heart-infusion broth (Difco) was used as the basal medium, with 2 % (w/v) Bacto agar (Difco) added to form heart-infusion agar, and 1 % (w/v) agar added to form soft agar. The agar media were routinely reinforced with 2×10^{-3} M-$Ca(NO_3)_2 . 4H_2O$ and 2×10^{-3} M-$MgSO_4 . 7H_2O$, except when 2×10^{-2} M-sodium citrate was added to form citrate agar.

Phage propagation and titration. Phages were propagated by the plate technique described by Williams & Rippon (1952) and titrated as described by these authors. Their notation was used to record the results of these titrations. Layer agar lawns as described by Adams (1950) were made where indicated in the text, by adding 0·1 ml. of a 4–6 hr. shaken culture of the appropriate staphylococcus to 2 ml. soft agar.

Production of the non-lysogenic staphylococcal strain 1363 (–). The non-lysogenic strain was isolated after irradiating the lysogenic strain 1363 (1363) with a heavy dose of ultraviolet radiation. A sample (0·1 ml.) of a 4 hr. shaken culture of the lysogenic strain (*c.* 10^8 organisms) was spread over the surface of a citrate agar plate and irradiated for 10 min. at a distance of 50 cm. from the centre of a 17 W. Westinghouse Sterilamp. The plate was then incubated at 37° until the surviving organisms had formed colonies. Citrate agar was used because non-lysogenic colonies on this medium are protected from infection with free phage. To identify the non-lysogenic colonies, the plate was replica-plated on to a layer agar lawn of indicator organisms (i.e. organisms sensitive to phage 1363). As described by Lederberg & Lederberg (1953), each lysogenic colony on the replica is surrounded by a zone of lysis, whereas non-lysogenic colonies are not. The latter were picked from the original citrate agar plate and shown to be sensitive to phage 1363.

We used staphylococcus 1363 (–) as the indicator organism in these experiments, since this strain was available, having been isolated by chance in the course of other work. Other staphylococci sensitive to 1363 phage might equally well have been used; two such strains are listed by Rountree (1949).

RESULTS

Presence of contaminating phage 1363 *in stock preparation of phage* 42 D.

Phage 42 D was propagated on staphylococcus 1363 (1363), and then titrated on this strain and on 1363 (42 D). The titrations of two preparations are shown in Table 1. The titration on the propagating strain shows the routine test dilution (the highest dilution giving confluent lysis) for each preparation, while the titration on 1363 (42 D) shows the level of contaminating phage present. It can be seen that when the first preparation is used at its routine test dilution the contaminating phage will be present in sufficient concentration to cause

Table 1. *Test for purity of stock preparations of staphylococcal typing phage* 42*D*

		Indicator staphylococcus	
		1363(1363)	1363(42D)
		Lytic effect	
	Phage dilution	For phage 42D	For contaminating phage 1363
Preparation A	Undiluted	Confluent lysis	Confluent lysis
	10^{-1}	Confluent lysis	++
	10^{-2}	Confluent lysis	++
	10^{-3}	Confluent lysis	+
	10^{-4}	++	−
	10^{-5}	+	−
	10^{-6}	±	−
Preparation B	Undiluted	Confluent lysis	++
	10^{-1}	Confluent lysis	±
	10^{-2}	Confluent lysis	−
	10^{-3}	++	−
	10^{-4}	+	−

a ± reaction, and that when this preparation is used undiluted the contaminating phage will give a confluent lysis reaction. When the second preparation is used at its routine test dilution the contaminating phage is diluted out, but when this preparation is used undiluted the contaminating phage will give a ++ reaction.

Production of the non-lysogenic staphylococcal strain 1363 (−)

The combined results of two experiments are shown in Table 2. It can be seen that the yield of non-lysogenic clones was extremely low, only two such clones being detected in 2214 survivors from *c.* 6×10^{-8} organisms.

Table 2. *Recovery rate of non-lysogenic strains of Staphylococcus following heavy ultraviolet irradiation*

Staphylococcus irradiated	No. of cocci irradiated	No. of colonies surviving	No. of colonies	
			Lysogenic	Non-lysogenic
1363 (1363)	*c.* 6×10^8	2214	2212	2

Isolation and propagation of a pure strain of phage 42D

Stock preparations of phage 42D are, as we have shown above, contaminated with phage 1363. From such a preparation a pure strain of phage 42D was obtained by single plaque isolation in the following way. First, the stock preparation was titrated in layered lawns of the non-lysogenic staphylococcal strain 1363 (−). In this titration both phage 42D and the contaminating phage 1363 formed similar plaques but, as can be seen from Table 1, most of the phage in the dilutions higher than 10^{-3} is likely to be phage 42D. An isolated plaque was therefore picked from the 10^{-6} dilution into 2 ml. broth. A loop of this suspension was spotted on to appropriate indicator staphylococci to confirm that this isolate was in fact phage 42D and the remainder of the suspension was carried through three single plaque isolations on the non-lysogenic staphylococcal strain. This pure strain of phage was propagated on the non-lysogenic staphylococci and then titrated on them and on staphylococcus 1363 (42D). It can be seen from Table 3 that there was no contaminating phage present.

Table 3. *Test for purity of pure phage 42D preparation*

	Indicator staphylococcus	
Phage dilution	1363 (–)	1363 (42D) (for contaminating phage 1363)
Undiluted	Confluent lysis	–
10^{-1}	Confluent lysis	–
10^{-2}	Confluent lysis	–
10^{-3}	++	–
10^{-4}	++	–
10^{-5}	±	–

DISCUSSION

The results presented here show that stock preparations of the staphylococcal typing-phage 42D are contaminated with phage 1363, and that it is possible to obtain a preparation free from such contamination by propagating a pure strain of phage 42D on artificially made non-lysogenic staphylococci.

As an alternative to this technique, a pure preparation of phage 42D can be obtained by inducing phage synthesis in staphylococcus 1363 (42D) by ultraviolet irradiation following the technique of Lwoff, Siminovitch & Kjeldgaard (1950). We found that Bacto tryptose (Difco) in 2% (w/v) solution reinforced with 2×10^{-3}M-$Ca(NO_3)_2 . 4H_2O$ was a suitable medium for this purpose. Although staphylococcus 1363 (42D) is inducible, it is of interest to record that the strain 1363 (1363) is only partially inducible and that this difference in the inducibility of the two prophages is maintained in the doubly-lysogenic strain 1363 (1363.42D). This method of obtaining a pure preparation of phage 42D is however of no practical advantage since in order deliberately to synthesize the staphylococcal strain 1363 (42D), it would be necessary first to obtain both non-lysogenic organisms and a pure preparation of phage 42D.

Serological techniques cannot be used to separate phage 42D from the contaminating phage 1363, for the two phages are serologically related. For example, an antiserum against the pure phage 42D with a K value (Adams, 1950) of 32 had a K value of 17 against the phage 1363. It is of interest to note that although these two phages are serologically related, there was no evidence of cross-protection by the corresponding prophages.

Finally, we have assumed throughout that staphylococcus 1363 (1363) is carrying only one prophage. If it should prove that this organism is carrying more than one prophage, then it would be necessary to eliminate these prophages in succession in order to obtain a completely non-lysogenic propagating strain.

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REFERENCES

Adams, M. H. (1950). Methods of study of bacterial viruses. *Meth. med. Res.* **2**, 1.

Lederberg, E. M. & Lederberg, J. (1953). Genetic studies of lysogenicity in *Escherichia coli*. *Genetics*, **38**, 51.

Lwoff, A., Siminovitch, L. & Kjeldgaard, N. (1950). Induction de la production des bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

Rippon, J. E. (1956). The classification of bacteriophages lysing staphylococci. *J. Hyg., Camb.* **54**, 213.

Rountree, P. M. (1949). The phenomenon of lysogenicity in staphylococci. *J. gen. Microbiol.* **3**, 153.

Rountree, P. M. (1956). Variations in a related series of staphylococcal bacteriophages. *J. gen. Microbiol.* **15**, 266.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.* **50**, 320.

Williams Smith, H. (1948). Investigation on the typing of staphylococci by means of bacteriophage. 1. The origin and nature of lysogenic strains. *J. Hyg., Camb.* **46**, 74.

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Funder, S. & Johannessen, S. (1957). *J. gen. Microbiol.* **17**, 117–119

The Membrane Filter as an Aid in the Cultivation and Identification of Fungi

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SUMMARY: A method of cultivating fungi on a membrane filter is described. The growth on the membrane appears to be more rapid than on solid media. After 2-3 days of growth a piece of the disk was dried and placed in immersion oil on a slide, thus rendering the membrane transparent and suitable for microscopic examination in transmitted light. The new method makes the use of plates, microcultures (slide cultures), etc., unnecessary. The mechanical breakage of the mycelial structures often encountered when species of mycelium have to be transferred to slides for microscopic examination is avoided. As only a small piece of the membrane is needed for microscopic examination, the growth on the membrane is allowed to continue as long as is required. Each time an inspection of the culture is required, a small piece of the disk is removed.

The 'molecular filter membrane' (MF) method has established itself as a valuable aid in sanitary bacteriology, especially in the bacteriological analysis of water, food products, beverages and other liquids. It has been shown by a number of workers that all micro-organisms which can be cultivated on a suitable medium can also be cultivated on the MF, when the MF is placed on that medium. After filtration of air, water or other fluids, the membrane may be stained, so as to make possible the direct microscopic examination of particles collected on the surface, including even such relatively large structures as fungal spores and pollen. Gordon, Ajello, Georg & Zeidberg (1952) reported on the use of MF in the detection of typical spores of *Microsporum gypseum* and *Histoplasma capsulatum* from soil and water. Such a procedure is, of course, effective only for those organisms which produce distinctive and identifiable microscopic structures. For the great majority of fungi, however, the spores are not so characteristic as to render direct identification possible. In view of the very delicate structure of the sporophores or other characteristic mycelial structures, this method appears to be of no value for identification purposes. In order to identify a fungus, the general procedure is to cultivate it on a suitable medium in Petri dishes, flasks, etc. When the cultural morphology is to be studied, a piece of aerial mycelium is removed and placed on a slide. Such a procedure often leads to mechanical destruction of the mycelial structure and makes identification difficult. In order to overcome this, a slide culture is often of great value. In certain cases, however, growth in a small volume of medium is not altogether typical. The slide culture must, therefore, be additional to, but not a substitute for, examination of the plate culture. The slide culture involves additional work and may be of no value in cases of fungi with no typical aerial mycelium, sporophores, etc. A cultivation method, without the use of plates, and providing the possibility of transferring the entire mycelial structure directly to the microscope without any mechanical disturbances, would be of great value in the identification. The MF method showed promise in this direction, and this was the reason why the study was undertaken.

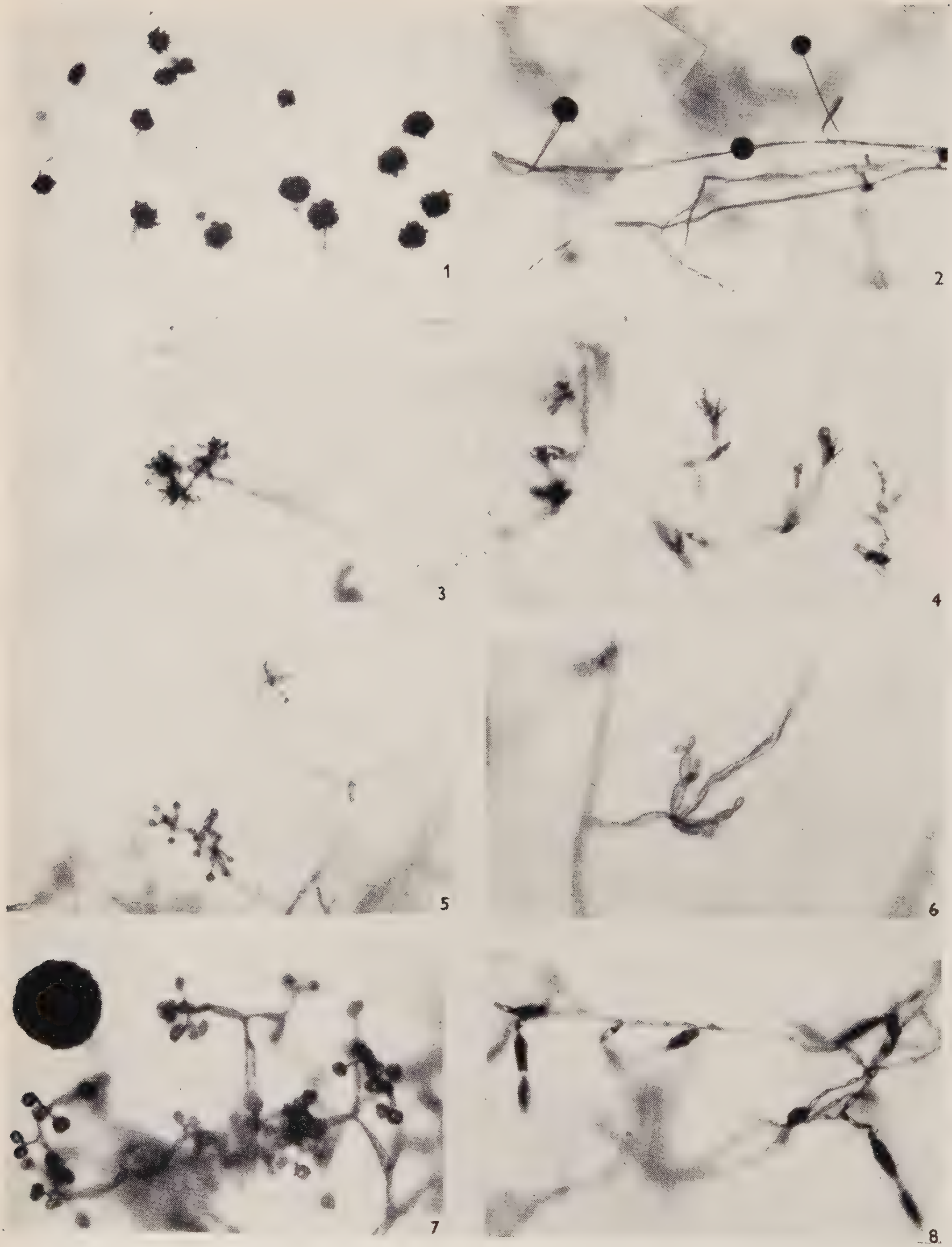
METHODS

Use was made of membrane filters manufactured by The Millipore Filter Corporation, U.S.A. and Membranfiltergesellschaft, Gøttingen. A sterile absorbent pad was placed at the bottom of a sterile Petri dish, standard size, and approximately 2 ml. sterile yeast water, double strength, was added, so that the pad was evenly moistened with the liquid. A sterile membrane filter was put on top, taking care to avoid the entrapment of air between the filter disk and the pad. Inoculation was made from cultures of fungi by streaking with a zig-zag motion over the surface of the moistened filter disk. The dish was incubated at room temperature for 2–3 days. When growth occurred, the entire MF, or a piece of it, was removed from the dish and dried at 50° for 20–30 min., or preferably at room temperature for 3–4 hr. A few drops of standard immersion oil ($n =$ approx. 1·50) were put on a slide and a piece of the dried MF carrying the culture placed on top. After a few seconds the membrane was rendered almost completely transparent (Jannasch, 1954) as soon as the pores were filled with the immersion oil, the membrane having the same refractive index as the immersion oil. The specimen was now ready for inspection by microscope, using transmitted light.

RESULTS

The following organisms were cultivated according to the method described: *Botrytis allii* (Centraalbureau voor Schimmelcultures); *Alternaria tenuis*, *Geotrichum candidum*, *Pullularia pullulans*, *Trichoderma lignorum* (Professor A. E. Traaen, The Norwegian Agricultural College); *Aspergillus flavus*, *Cladosporium* sp., *Monilia sitophila* (Cand. pharm. O. Clausen, Institute of Pharmacy, University of Oslo); *Syncephalastrum cinereum*, *Thamnidium elegans* (Botanical Laboratory, University of Oslo); *Mucor racemosus*, *Penicillium roqueforti*, *Rhizopus nigricans* (Dr S. Funder, Institute of Pharmacy, University of Oslo).

In many cases only 2 days of growth were sufficient to reveal characteristic details for identification of the organisms. This is in accordance with Orlando & Bolduan (1953) and Tsuneishi & Goetz (1953) who observed that the growth in many cases was more rapid on MF than on solid media. When 2 days growth was not sufficient, the MF was kept for another day or more. At each inspection a new piece of the MF was treated as described, while the growth was allowed to continue on the remaining part of the disk. In Pl. 1 photomicrographs of some of the fungi which were studied are shown.



S. Funder and S. Johannessen—The growth of fungi on membranes. Plate 1

(*Facing p.* 119)

REFERENCES

GORDON, M. A., AJELLO, L., GEORG, L. K. & ZEIDBERG, L. D. (1952). *Microsporum gypseum* and *Histoplasmac apsulatum* spores in soil and water. *Science*, **116**, 208.

JANNASCH, H. W. (1954). Beitrag zur Methode der direkten mikroskopischen Untersuchung von Mikroorganismen auf Membranfiltern: Über die Aufhellung der Filter. *Zbl. Bakt.* (1. *Orig.*), **161**, 225.

ORLANDO, M. D. & BOLDUAN, O. E. A. (1953). The application of the membrane filter to a variety of pathogenic bacteria and Fungi Imperfecti. *Bact. Proc.* (53rd. Gen. Meet.), p. 26.

TSUNEISHI, N. & GOETZ, A. (1953). Growth development and incubation of bacterial cultures on molecular filter membranes. *Bact. Proc.* (53rd Gen. Meet.), p. 26.

EXPLANATION OF PLATE

Fig. 1. *Aspergillus flavus.* ×75.
Fig. 2. *Mucor racemosus.* ×75.
Fig. 3. *Botrytis alii.* ×250.
Fig. 4. *Penicillium roqueforti.* ×250.
Fig. 5. *Trichoderma lignorum.* ×250.
Fig. 6. *Cladosporium* sp. ×500.
Fig. 7. *Thamnidium elegans.* ×250.
Fig. 8. *Alternaria tenuis.* ×250.

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Kirkham, D. S. (1957). *J. gen. Microbiol.* 17, 120–134

Studies of the Significance of Polyphenolic Host Metabolites in the Nutrition of *Venturia inaequalis* and *Venturia pirina*

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SUMMARY: Extracts of the polyphenolic fractions of the water-soluble metabolites (that is, the complex mixtures of plant phenols extracted by ethyl acetate) were prepared from apple and pear varieties characterized by different degrees of resistance to the scab pathogens. Qualitative differences were chiefly interspecific and extracts of resistant varieties did not contain major components lacking in those of less-resistant varieties of the same species. Cultural reactions of distinct clones of each pathogen to the extracts, in the presence of various basal media, were observed. Growth and sporulation were inhibited independently by extracts of less-resistant as well as resistant host varieties. The clones were not equally susceptible, those of *Venturia inaequalis* showing relationships between inhibition of sporulation by host polyphenols and their varietal host ranges. Fluctuations in pathogenicity of a clone of *V. pirina* during storage in culture with periodic re-isolation from Williams pear were reflected in its reactions to the extracts. Both pathogens were capable of decomposing the polyphenols. The results suggested that qualitative and quantitative variations in the polyphenolic host metabolites, including differences in their relative proportions, in relation to nitrogenous and other nutritional factors, are of potential significance in the determination of pathogenicity and varietal resistance.

A study of distinct clones of *Venturia inaequalis* (Cke.) Wint. and *V. pirina* Aderh., the causal fungi of apple and pear scab, respectively (Kirkham, 1957) has shown that interspecific and interclonal differences in pathogenicity and host range are not necessarily associated with corresponding differences in nutritional requirements. Clones of *V. inaequalis* which were pathogenic on the greatest number of apple varieties were the most stable in artificial culture, but no other indications were obtained of relationships between the reactions of either pathogen in natural and artificial environments. These observations, and the work of Wiltshire (1915), Johnstone (1931) and Rudloff (1934), point to the importance of considering all groups of host metabolites, including the plant phenols, as factors in the metabolism of *Venturia* species and in the determination of their host relations. Bradfield & Flood (1949) showed that the main qualitative differences between the water-soluble constituents of apple and pear shoots lie in the polyphenolic fractions (that is, the complex mixtures of plant phenols extractable by ethyl acetate). In the present communication polyphenolic fractions of apple and pear varieties characterized by different degrees of resistance are compared, and the results of cultural studies of the possible significance of these and other host metabolites in the nutrition of the pathogens are given. Some results of this study have already been briefly reported (Kirkham, 1954).

METHODS

The first series of extracts was prepared from young leaves of the apple varieties Cox's Orange Pippin and Miller's Seedling on Malling II rootstocks, and of the pear varieties Williams' Bon Chrétien and Conference on Quince A rootstocks. Young leaves were defined as all those younger than, and including the first fully unrolled leaf, below a shoot tip; the polyphenolic fractions were obtained by direct extraction of the macerated leaves with ethyl acetate (Bradfield in 1951). A second series of extracts was prepared by the same method from mature leaves of the same varieties (mature leaves being defined as those of maximum size on a shoot). Considerable enzymic browning of the pulp, particularly of the pear material, occurred during extraction and resulted in some decrease in yield. Later, a third series of extracts was prepared from young leaves of the same varieties when an extraction method similar to that described by Bradfield & Flood (1949) was used. Each sample of leaf material was macerated in aqueous methanol (80 %, v/v), and the pulp then thoroughly washed with fresh solvent until the washings were colourless. Most of the methanol was removed by distillation under reduced pressure, and the residual aqueous solution separated from the green chlorophyll sludge by centrifugation. This solution was then extracted with ethyl acetate to separate the polyphenolic fraction from the other water-soluble leaf constitutents. Only very slight browning of the apple- and pear-leaf pulp was observed. The three extracts prepared from each variety were numbered 1, 2 and 3. Pure samples of phloridzin, arbutin, chlorogenic acid, *iso*chlorogenic acid and the polyphenolic fraction of the rootstock SV411 were supplied by the Biochemistry Section of East Malling Research Station. Chromatograms were run by upward displacement on Whatman no. 1 paper at 21° using as solvent mixture *n*-butanol/acetic acid/water (63/10/27, v/v; Campbell, Work & Mellanby, 1951). After solvent removal the chromatograms were examined under ultraviolet light before and after treatment with ammonia, and were subsequently sprayed with diazotized *p*-nitroaniline in sodium acetate solution (Bradfield, 1951; private communication).

Six clones of *Venturia inaequalis* and five of *V. pirina* (described in detail by Kirkham, 1957) were used in these studies. The apple or pear variety sources of the *Venturia* clones, arranged in increasing order of field-resistance, and their host ranges are indicated in Table 1. During the work the clones of *Venturia* spp. were stored under paraffin oil and were periodically re-isolated as single conida either from the stock cultures (re-isolation 2) or from artificially inoculated shoots (re-isolations 1 and 3). The time intervals between primary isolation (P.I.) from field-infected material and the re-isolations were as follows: P.I.-re-isolation 1, 24 months (clones E1 and D2) and 12 months for the remaining clones; re-isolations 1–2, 11 months; 2–3, 7 months.

The clones of *Venturia* spp. were grown on filter-paper cylinders standing in filter-sterilized liquid media; degree of sporulation was estimated by a turbidimetric method in which the readings were directly proportional to the number of conidia produced (Kirkham, 1956). The cultures were incubated to

Table 1. *Varietal sources and host ranges of distinct clones of* Venturia inaequalis *and* V. pirina

Organism	Clone	Apple (for *V. inaequalis*) or pear (for *V. pirina*) variety source*	Extent of host range.† Number of varieties attacked
V. inaequalis	A 26	Cox's Orange Pippin	3
	A 6	Worcester Pearmain	5
	A 7	Worcester Pearmain	6
	A 20	Laxton's Superb	7
	E 1	Edward VII	8
V. pirina	P 16	Williams' Bon Chrétien	6
	P 17	Williams' Bon Chrétien	5
	P 6	Laxton's Superb	7
	D 2	Durondeau	6
	81′	Conference	3

* Varieties arranged in increasing order of field resistance.

† Number of sporulating lesion reactions resulting from inoculation of nine apple varieties with each clone of *V. inaequalis* and eight pear varieties with each clone of *V. pirina*.

maturity at 18°, that is until it seemed that sporulation had reached a maximal value in the treatment. Growth was visually assessed in the following categories: 0 = none or trace; 1 = thin and inhibited; 2 = normal but suboptimal; 3 = optimal. The polyphenolic host-metabolites were added to and dissolved in various basal media (at pH 5·6) immediately before sterilization by filtration. Malt-extract (10 %, w/v) medium, a partially defined medium and modifications of the latter were used as basal media. 'NC' refers to a partially defined medium of the following composition: glucose, 1·5 g.; enzymic digest of casein 0·35 g.; thiamine 30μg.; $MgSO_4.7H_2O$ 0·05 g.; KH_2PO_4 0·15 g.; trace elements as used by Leben & Keitt (1948); all in 100 ml. distilled water. Variations in the composition of this medium are indicated, for example: $\frac{1}{3}$NC = casein digest at one-third of the concentration above; N3C = glucose at three times the normal concentration; NC.20T = trace elements at twenty times the normal concentration. The media also contained traces of malt-extract from the unwashed inoculum and KCl resulting from the adjustment of pH value.

Each treatment was applied in duplicate and the turbidimetric estimate of degree of sporulation expressed as the geometric mean of a pair of observations. Two means differed significantly (5 % level of significance) when their ratio exceeded 1·45.

RESULTS

Comparison of polyphenolic-fraction extracts of resistant and less resistant varieties of apple and pear

Miller's Seedling and Cox's Orange Pippin were compared as examples of highly scab-resistant and less-resistant apple varieties, respectively, and Conference and Williams' Bon Chrétien as resistant and less-resistant pear varieties. The complexity of the three extracts prepared from each variety (2 % aqueous solutions) is illustrated by the twelve chromatograms shown in

Pl. 1, fig. 1. The most outstanding differences were those between the apple and pear extracts, as noted by Bradfield & Flood (1949). The major component of the apple fractions is phloridzin (3); the major components of the pear fractions are arbutin (1), *iso*chlorogenic acid (5) and chlorogenic acid (2) which also occurs as a minor constituent of the apple extracts. The aglycones phloretin (4) and hydroquinone (6) of phloridzin and arbutin, respectively, were present in the first and second series of extracts, probably as artefacts because of the extraction method used.

It is clear that the major components of the polyphenolic fractions of resistant and less-resistant varieties of apple and pear, and of young and mature leaves, are the same. There are, however, indications of quantitative differences and of differences between the relative proportions of these components. Chlorogenic acid was present at higher concentrations in the Miller series than in the Cox series, and extracts of mature leaves contained more than those of young leaves. The Conference fractions contained more arbutin, chlorogenic acid and *iso*chlorogenic acid than the Williams fractions, with the exception that there was more *iso*chlorogenic acid in Williams 3 than in Conference 3. The ratios between the amounts of chlorogenic acid and *iso*chlorogenic acid in each pear extract appeared to differ, being probably higher, for example, in Conference 3 than in Conference 1. Qualitative as well as quantitative differences were apparent between minor components. Preliminary evidence indicates the presence of further depside-like compounds in pear, of which there were more in Williams than in Conference. The Miller extracts contained more compounds than the Cox extracts and young leaves contained more polyphenolic compounds than mature leaves.

It is now known that the extraction techniques used were not exhaustive and that preferential extraction of some compounds probably occurred. Thus comparison of total yields of polyphenolic fraction extracts with weights of fresh material was not possible, and the quantitative variations described above cannot be regarded as definite indications of differences between varieties. It was observed however that the Miller 3 yield (per unit fresh weight of leaves) was considerably higher than that of Cox 3 and it is therefore possible that the Miller leaf-sample contained more phloridzin and chlorogenic acid than the Cox leaf-sample.

The activity of extracts cannot be related to the major components alone owing to the qualitative variation among the minor components, and the significance of single compounds must be assessed with care owing to possible synergistic effects between them in mixtures.

Reactions of the pathogenic Venturia *spp. to polyphenolic host-metabolites*

Table 2 shows that polyphenolic host-metabolites inhibited sporulation and to a lesser extent growth of the pathogens in the NC basal medium. The clones were not equally susceptible to this inhibition but, excepting the reactions of *Venturia pirina* clone D2, extracts of resistant and less-resistant varieties, showed similar activities. This observation supports the chemical evidence of similarities between the extracts and does not suggest the

Table 2. *Effects of polyphenolic host metabolites on sporulation and growth of* Venturia inaequalis *clones E*1, *A*26, *A*20 *and A*6 *and* V. pirina *clones D*2, 81′, *P*16 *and P*6

Test 24: 9 months after re-isolation 1. Basal medium NC throughout.

Additions to basal medium NC (%, w/v)	*V. inaequalis*, sporulation*				Growth category†				Days incubation			
	E1	A26	A20	A6	E1	A26	A20	A6	E1	A26	A20	A6
—	0·141	0·112	0·085	0·132	2	2	2	2	17	14	24	19
Cox 3 extract; 0·5	0·102	0·007	0·051	0·055	2	2	2	2	17	14	24	19
Miller 3 extract; 0·5	0·117	0·004	0·050	0·087	2	2	2	2	17	14	24	19
Chlorogenic acid; 0·1	0·098	0·017	0·085	0·025	2	1	1	1	17	19	24	19
	V. pirina, sporulation*				Growth category†				Days incubation			
	D2	81′	P16	P6	D2	81′	P16	P6	D2	81′	P16	P6
—	0·059	0·019	0·021	0·038	2	2	2	2	19	19	17	19
Williams 3 extract; 0·5	0·050	0·024	0·001	0·014	2	2	1	2	19	19	19	19
Conference 3 extract; 0·5	0·026	0·016	0·001	0·016	2	2	1	2	19	19	19	19
Chlorogenic acid; 0·1	0·056	0·013	0·004	0·010	2	2	2	2	19	19	19	19

* Spekker drum reading of conidial suspension directly proportional to the number of conidia produced.

† 0 = none or traces; 1 = thin and inhibited; 2 = normal but suboptimal; 3 = optimal.

occurrence of resistance factors which are lacking in less-resistant varieties such as Cox's Orange Pippin and Williams' Bon Chrétien. The four clones of *V. inaequalis* showed relationships between their host varietal ranges (Table 1) and their susceptibility to inhibition. Thus clone A 26, which had the narrowest host range, was the most susceptible and clone E1, isolated from the resistant variety Edward VII and having the widest host range, was the least inhibited. No such relationships were observed within the clones of *V. pirina.* The results suggest there may be differences in the significance of polyphenolic host-metabolites in the metabolism of the two pathogens.

Chlorogenic acid was a highly active component of the extracts. Its inhibitory effects at 0·1 % (w/v) closely followed those of the extracts, being sometimes rather more and sometimes rather less active than the extracts at 0·5 %. A further test gave some indication that a mixture of chlorogenic acid and *iso*chlorogenic acid was more active against E1 than either compound alone at the same concentration. This indication of synergism between the known depsides as toxic factors indicates the potential importance of any variation in the relative proportions of the polyphenolic host-metabolites under natural conditions.

Table 3. *Inhibition of* Venturia inaequalis *clones* E1 *and* A26, *by the polyphenolic fraction of the apple rootstock* SV 411

Test 23: basal medium NC throughout.

Additions to basal medium NC (%, w/v)	Sporulation		Growth category		Days incubation	
	E1	A26	E1	A26	E1	A26
—	0·214	0·269	2	2	25	32
SV411 extract; 0·5	0·174	0·044	2	1	25	32
SV411 extract; 2·0	0·001	0·001	1	1	29	32
SV411 extract; 5·0	<0·001	<0·001	0	0	29	32

The effects of increasing the amount of a host extract in medium NC, on *Venturia inaequalis* are shown in Table 3. Clones E1 and A26, which differed widely in sensitivity to the Cox 3 and the Miller 3 extracts (Table 2), showed similarly contrasted reactions to the extract of SV411 at 0·5 %. At concentrations of 2 and 5 %, where growth inhibition became marked, this difference was no longer apparent. It seems that these clonal reactions are only visible at low extract concentrations and then only when sporulation is measured as the main cultural response. Host resistance *in vivo*, after successful infection, is often indicated by the production of lesions bearing no or few conidia, and a demonstration of similar effects in culture may therefore be relevant to the question of host resistance.

Sporulation and growth of *Venturia pirina* clone D2 were also strongly inhibited when the concentration of the Conference 3 host-extract was increased beyond 0·5 % (Table 4). Opposite effects were, however, observed with apple extracts at the same and higher concentrations; marked stimulation of sporulation occurred but the conidia were abnormal in shape, being similar to those of *V. inaequalis*. Phloridzin (0·2 %) alone was also highly stimulatory of sporulation and may therefore account for this degree of activity, though it was apparently decreased in the presence of the other apple extract components. Arbutin (0·2 %), the main pear glycoside, was inactive as a stimulator or as an inhibitor of *V. pirina.* Table 4 also demonstrates a striking difference between the reactions of *V. pirina* clone D2 to the Conference 1 and 3 extracts. While Conference 3 was strongly inhibitory at 1 %, Conference 1 at 2 % increased the length of the lag phase and slightly inhibited growth, but sporulation was greatly increased and normal conidia were produced. This difference must be explicable in terms of chemical composition (Fig. 1), though the modes of action of the various components are as yet unknown. The main difference between the two extracts was that Conference 3 contained arbutin as a major constituent, while in Conference 1 the major constituent was hydroquinone, as a result of the extraction method. Attention has been drawn by Siebs (1955) to the possible significance of hydroquinone in the host relations of *V. pirina,* though in contrast to the present result he suggested that a high concentration of hydroquinone in the host tissues might induce resistance to the pathogen.

Table 4. *Inhibition and stimulation of sporulation of* Venturia pirina *clone* D2 *by polyphenolic fractions and major components*

Test 25: 9 months after re-isolation 1; test 22: 5 months after re-isolation 1. Basal medium NC throughout.

Additions to basal medium NC. Extracts, etc. (%, w/v)	Sporulation	Growth category	Days incubation
	Test 25		
—	0·036	2	19
Conference 3; 0·5	0·018	2	19
Conference 3; 1·0	<0·001	1	19
Miller 3; 0·5	0·186	2	19
Miller 3; 1·0	0·229	2	19
	Test 22		
—	0·028	2	12
Conference 1; 2·0	0·117	1–2	17
Arbutin; 0·2	0·030	2	12
Cox 1; 2·0	0·105	1–2	17
Phloridzin, 0·2	0·229	2	12

Effects of basal media on reactions of Venturia inaequalis

Reactions of *Venturia inaequalis* clones E1 and A20, in the presence of three different basal media, to Cox 3 extract (1 %) and to phloridzin (0·2 %) are illustrated in Table 5. Clone E1 was not inhibited by either extract when added to 10 % malt-extract medium, but sporulation of clone A20 in 10 % malt-extract medium was considerably decreased by Cox 3 extract. With medium NC the sporulation of both clones was decreased by each addition, though inhibition of clone A20 was more marked. The third basal medium 3NC, containing three times the normal amount of casein digest, increased the sporulation of clone A20, but the degree of inhibition of both clones by phloridzin (0·2 %, w/v) remained the same as in NC medium. Cox 3 extract, however, was much more inhibitory in these conditions, particularly to clone E1. Thus inhibition was less marked in the presence of a highly-concentrated and complex natural medium, but an increase of the casein digest concentration as in NC did not decrease the degree of inhibition and even had the opposite effect on reactions to the polyphenolic fraction of Cox. Phloridzin was evidently an active component of this fraction, but as the 1 % solution of Cox 3 extract probably contained phloridzin at a concentration higher than 0·2 %, this compound was apparently less active in the mixture.

Table 5. *Effects of three different basal media on reactions of* Venturia inaequalis *clones* E1 *and* A20 *to polyphenolic host constituents*

Test 34: 18 months after re-isolation 1 (7 months after re-isolation 2).

		Sporulation		Growth category		Days incubation	
Basal medium	Additions (%, w/v)	E1	A20	E1	A20	E1	A20
10 % Malt-extract	—	0·641	0·100	3	3	17	14
10 % Malt-extract	Cox 3 extract; 1·0	0·575	0·035	3	3	17	14
10 % Malt-extract	Phloridzin; 0·2	0·646	0·129	3	3	17	14
NC	—	0·257	0·135	2	2	19	17
NC	Cox 3 extract; 1·0	0·126	0·024	2	2	19	17
NC	Phloridzin; 0·2	0·115	0·081	1	2	19	17
3NC	—	0·269	0·240	2	2	19	17
3NC	Cox 3 extract; 1·0	0·003	0·014	2	2	19	17
3NC	Phloridzin; 0·2	0·123	0·174	1	2	19	17

Table 6 shows that in test 36 Conference 3 extract (0·5 %) was strongly inhibitory to *Venturia inaequalis* clone E1 and considerably less active against clone A7, in medium NC. Increase of the casein digest concentration in this medium decreased inhibition of clone E1, the differences between the two clones being no longer apparent. These results show, first, that a polyphenolic-fraction of the pear inhibited *V. inaequalis*, *in vitro*, in contrast with the lack of inhibition of *V. pirina* by an apple extract (Table 4) and, secondly, that an increase of nitrogen in the basal medium decreased this inhibition, though, in the same conditions inhibition by Cox 3 extract was increased (Table 5). The pear extracts were, in general, more toxic in culture to both pathogens than were the apple extracts. The higher content of chlorogenic acid and *iso*chlorogenic acid in the former may partly account for this result. Lack of cross-infection does not seem to be explicable in terms of the cultural reactions to polyphenolic extracts which have been observed.

Venturia inaequalis clones E1 and A7 showed a high degree of tolerance towards trace element concentrations up to twenty times those in NC medium (medium NC.20T). In these conditions clone E1 was much less inhibited by Cox 3 extract than in test 34 (Table 5), but the reaction of the more susceptible clone A7 was unaltered. In test 36 a higher nitrogen concentration again increased the degree of inhibition of clone E1 by Cox 3 extract. The reactions of clones A6 and A20 to Cox 3 extract were also observed in media NC.20T and 2NC.20T in this test. In contrast to the behaviour of clone E1, clone A6

Table 6. *Effects of basal media and storage under paraffin oil on reactions of* Venturia inaequalis *clones* E1 *and* A7, *to polyphenolic fractions of apple and pear.*

Test 36: 2 months after re-isolation 3; test 37: 4 months after re-isolation 3.

		Sporulation		Growth category		Days incubation	
Basal medium	Additions (%, w/v)	E1	A7	E1	A7	E1	A7
		Test 36					
NC	—	0·398	0·302	2	2	21	26
NC	Conference 3 extract; 0·5	0·001	0·162	1	2	21	26
2NC	—	0·417	0·229	2	2	21	26
2NC	Conference 3 extract; 0·5	0·224	0·132	3	3	21	26
NC.20T	—	0·398	0·123	2	2	21	26
NC.20T	Cox 3 extract; 1·0	0·316	0·005	2	3	21	26
2NC.20T	—	0·437	0·148	2	2	21	26
2NC.20T	Cox 3 extract; 1·0	0·209	0·002	1	2	21	26
		Test 37					
NC	—	0·251	—	2	—	17	—
NC	Conference 3 extract; 0·5	0·195	—	3	—	17	—
NC	Cox 3 extract; 1·0	0·027	—	1	—	17	—

was considerably less inhibited with the increased nitrogen concentration, and clone A20 (though much less susceptible to Cox 3 extract than was clone A7) also showed no reaction to the increased nitrogen concentration.

In general, a high degree of correlation was observed between the results of similar tests carried out on separate occasions. However, test 36 made 2 months after re-isolation 3, showed that clone A7 was considerably more resistant and clone E1 less resistant to Conference 3 extract than in earlier tests before this re-isolation. Two months later (test 37) clone E1 reacted to Conference 3 extract as had clone A7 in test 36 and was also very susceptible to Cox 3 extract (1 %) in NC medium. It is now thought that, after re-isolation 3, clone E1 was, while more sensitive to Conference 3 extract, less susceptible to Cox 3 extract (compare test 34), a situation which changed again during further storage of the clones. Unfortunately the factors of increased trace element concentration and possible temporary adaptation to the host substrate, indicated by the reactions to Cox 3 extract, cannot be separated.

Effects of storage under paraffin oil and re-isolation on reactions of Venturia pirina

Tables 2, 4, 7 and 8 show results obtained with *Venturia pirina* clone D2, during 17 months' storage under paraffin oil. At the beginning and at the end of this period, clone D2 and other clones were re-isolated from artificially inoculated shoots of Williams' Bon Chrétien pear (re-isolations 1 and 3). Eleven months after re-isolation 1 (before test 28, Table 7) the clones were

Table 7. *Inhibition and stimulation of* Venturia pirina *clone* D2 *by polyphenolic metabolites with two basal media*

Test 28: 12 months after re-isolation 1 (1 month after re-isolation 2); test 29: 14 months after re-isolation 1 (3 months after re-isolation 2).

Basal medium	Additions (%, w/v)	Sporulation	Growth category	Days incubation
	Test 28			
NC	—	0·025	2	18
NC	Chlorogenic acid; 0·2	0·031	2	18
NC	Chlorogenic + *iso*chlorogenic acid; 0·2	0·008	2	18
NC	Phloridzin; 0·2	0·135	1	18
	Test 29			
NC	—	0·025	2	17
NC	Conference 3 extract; 1·0	0·013	1–2	28
NC	Arbutin; 0·4	0·011	2	17
NC	Cox 3 extract; 5·0	$<0·001$	1	28
NC	Phloridzin; 0·2	0·059	2	17
3N3C	—	0·023	2	17
3N3C	Conference 3 extract; 1·0	0·004	2	17
3N3C	Arbutin; 0·4	0·014	3	17
3N3C	Cox 3 extract; 5·0	0·038	1–2	24
3N3C	Phloridzin; 0·2	0·038	2	17

transferred as single conidia from the stock cultures (re-isolation 2). Table 7 shows that test 28 gave some indication of increased sporulation of clone D2 in the presence of chlorogenic acid which was not apparent in test 24 (Table 2). In the same conditions crude *iso*chlorogenic acid containing chlorogenic acid decreased the sporulation of clone D2 considerably. Phloridzin was less stimulatory than in test 22; 2 months later, this activity was again significantly decreased (test 29). The latter test now showed Conference 3 extract to be less active than it had been when tested 5 months previously and Cox 3 extract to be strongly inhibitory at 5% (w/v). Threefold increases of the glucose and casein digest concentrations in the basal medium resulted in normal growth with Conference 3 extract, but fewer conidia were produced and in these conditions 5% (w/v) Cox 3 extract did not decrease the degree of sporulation. Arbutin (0·4%, w/v) was slightly inhibitory in both media. These results show that an apple extract may be toxic to *V. pirina* at a high concentration and also suggest two distinct parallels with the reactions of *V. inaequalis*, where increased casein digest concentration increased inhibition by the host extract (Cox 3) and decreased inhibition by Conference 3 extract (Tables 5 and 6).

Table 8 illustrates results of test 33, 17 months after re-isolation 1, which are in distinct contrast with those of the earlier tests 22 and 25. Conference 3 extract (0·5%, w/v) here increased sporulation of clones D2 and P17, while Conference 1 extract (2%, w/v) was almost completely inhibitory. The use of medium NC.20T induced only minor differences in these reactions except that clone P17 no longer produced more conidia in the presence of Conference 3 extract. These results were confirmed in test 35 where sporulation of clone 81′ was not increased by Conference 3 extract. An increase of the nitrogen concentration to that of medium 3NC.20T masked the stimulation of clone D2 by Conference 3 extract, which now inhibited clone 81′. These results show a trend similar to that shown in test 29 where inhibition of sporulation by Conference 3 extract was increased on medium 3N 3C, but where the final growth category was somewhat higher.

Table 8. *Effects of basal media and storage under paraffin oil on reactions of* Venturia pirina, *clones* D2, P17 *and* 81′, *to the Conference* 1 *and* 3 *extracts*

Test 33: 17 months after re-isolation 1 (6 months after re-isolation 2); test 35: 18 months after re-isolation 1 (7 months after re-isolation 2).

Basal medium	Extracts added (%, w/v)	Sporulation		Growth category		Days incubation	
		D2	P17	D2	P17	D2	P17
		Test 33					
NC	—	0·035	0·007	2	2	20	20
NC	Conference 3; 0·5	0·066	0·019	2	2–3	20	20
NC	Conference 1; 2·0	<0·001	<0·001	0	0	30	30
NC.2OT	—	0·039	0·006	2	2	20	20
NC.2OT	Conference 3; 0·5	0·085	0·009	2	2–3	20	20
NC.2OT	Conference 1; 2·0	<0·001	<0·001	0	0	30	30
		Sporulation		Growth category		Days incubation	
		D2	81′	D2	81′	D2	81′
		Test 35					
NC.2OT	—	0·034	0·042	2	2	18	18
NC.2OT	Conference 3; 0·5	0·091	0·043	2	2	18	18
NC.2OT	Conference 1; 1·5	<0·001	<0·001	1	0	30	30
3NC.2OT	—	0·065	0·048	2	2	18	18
3NC.2OT	Conference 3; 0·5	0·068	0·028	2–3	2–3	18	18
3NC.2OT	Conference 1; 1·5	<0·001	<0·001	1	1	30	30

Table 9 shows, first, the results of test 36 which was carried out 2 months after re-isolation 3, from Williams' Bon Chrétien. Reactions to Conference 1 and 3 extracts were similar to those in tests 22 and 25, 5 and 9 months after the first re-isolation respectively. Control sporulation of clone D2 was to a higher degree than in test 35, Conference 3 extract was slightly inhibitory, and Conference 1 extract was stimulatory after an increased lag phase, and with a decrease in final amount of growth. Increase of casein digest in the basal medium slightly increased growth in the presence of Conference 3 extract but had no effect on sporulation. Test 37, 4 months after re-isolation 3, yielded results comparable with those of test 33, 17 months after re-isolation 1. Conference 3 extract (0·5% w/v) again markedly increased sporulation, while Conference 1 extract (1% w/v) was intensely inhibitory. Chlorogenic acid increased sporulation of clone D2, thus confirming the indication in test 28 (Table 7).

There appears to be a definite relationship between the cultural reactions described above, particularly to the Conference 1 and 3 extracts, and suc-

Table 9. *Effects of re-isolation of* Venturia pirina *from artificially-inoculated shoots of Williams, on reactions to polyphenolic metabolites*

Test 36: 2 months after re-isolation 3; test 37: 4 months after re-isolation 3.

Basal medium	Additions (%, w/v)	Sporulation		Growth category		Days incubation	
		D2	P16	D2	P16	D2	P16
		Test 36					
NC	—	0·076	—	2	—	19	—
NC	Conference 3 extract; 0·5	0·063	—	2	—	19	—
NC	Conference 1 extract; 1·0	0·141	—	1–2	—	26	—
2NC	—	0·078	—	2	—	19	—
2NC	Conference 3 extract; 0·5	0·056	—	2–3	—	19	—
2NC	Conference 1 extract; 1·0	0·129	—	1–2	—	26	—
		Test 37					
NC	—	0·055	0·010	2	2	19	17
NC	Conference 3 extract; 0·5	0·132	0·035	2	2	19	17
NC	Conference 1 extract; 1·0	0·001	0·001	1	0	25	25
NC	Cox 3 extract; 1·0	0·339	0·275	2	2	19	17
NC	Chlorogenic acid; 0·5	0·087	0·030	2	2	19	17

cessive re-isolations of the pathogen from Williams. A gradual change occurred during the 18 months' storage under paraffin oil between re-isolations 1 and 3, which was not influenced by the mono-conidial transfer of the stock cultures. The sudden reversal after re-isolation 3 and the rapid return to the type of reaction observed before it are also noteworthy. It was shown by Kirkham (1957) that the pathogenicity of *Venturia pirina* may decline more rapidly during storage in culture than the pathogenicity of *V. inaequalis*, and that the rate of this decline increased after each successive re-isolation from Williams. There seems to be a definite parallel between the stability of the pathogenic capabilities of *V. pirina* on Williams and reactions to polyphenolic host constituents, which strongly suggests that the reactions of a given clone to these compounds, in relation to other nutritional factors, may be important in the determination of pathogenicity.

Decomposition of polyphenolic host metabolites by Venturia inaequalis *and* V. pirina *in artificial culture*

Intense colour reactions resulted from the presence of the extracts and components in the various basal media during the lag phases of growth of both pathogens. Chromatographic examination of media containing single metabolites showed that they were decomposed with the formation of high molecular weight products. Fig. 2 shows chromatograms of stages in the decomposition of arbutin and chlorogenic acid by *Venturia inaequalis*, clones E1 and A7. Spots visible in ultraviolet light are outlined in pencil. Table 10 shows the comparative amounts of decomposition visible on the chromatograms. Chlorogenic acid was destroyed more rapidly than arbutin, clone E1 was more active than clone A7, and increase of casein digest concentration

Table 10. Venturia inaequalis. *Decomposition of arbutin and chlorogenic acid in two basal media by clones* E1 *and* A7 (*see Pl.* 1, *Fig.* 2)

Chromatogram no.	Metabolite	Clone of *V. inaequalis*	Basal medium	Period of incubation (days)	Degree of decomposition*
1	Arbutin; 0·01 M	E1	NC	8	+ + + +
2		(Uninoculated control)	NC	8	Trace
3		A7	NC	8	+ +
4		E1	3 NC	8	+ + +
5		(Uninoculated control)	3 NC	8	Trace
6		A7	3 NC	8	+
7	Chlorogenic acid; 0·01 M	E1	NC	5	+ + + +
8		(Uninoculated control)	NC	5	Trace
9		A7	NC	5	+ +
10		E1	3 NC	5	+ + +
11		(Uninoculated control)	3 NC	5	Trace
12		A7	3 NC	5	+ +

* + + + + indicates maximal decomposition of metabolite.

in the basal medium slowed down the rate of decomposition to some extent. These observations may be compared with the facts that clone A7 was more susceptible to inhibition by the extracts than clone E1, and that higher concentrations of nitrogen sometimes increased the inhibitory activities of the extracts. The chromatograms showed that during the course of decomposition, various intermediates were present in the basal media. One of these was identified as hydroquinone, temporarily present as a result of the destruction of arbutin by *V. pirina*. When decomposition was complete the metabolites and intermediates finally disappeared completely, leaving only compounds of high molecular weight which remained on the starting line. Only very slight breakdown occurred during incubation of the uninoculated controls. Decomposition of arbutin also occurred in the presence of a culture filtrate of clone E1, thus demonstrating the exogenous activity of an enzyme system.

These results show that both *Venturia inaequalis* and *V. pirina* produce polyphenoloxidase enzyme systems capable of destroying compounds inhibitory to normal development. Nevertheless, it was found that, even after complete breakdown, inhibition of sporulation was frequently recorded at the end of the incubation period. This may have been due to changes in the metabolism of the fungi induced by the presence of these compounds or to the activity of undetected intermediates.

DISCUSSION

It is clear from the foregoing experiments that polyphenolic host metabolites are of considerable significance in the metabolism of the pathogens in artificial culture, and the results suggest the possibility of their operation as resistance factors. In both cases there are indications of optimal balance mechanisms between the polyphenols of the host and the nitrogenous constituents of the basal media, though other nutritional factors are also clearly of importance. These mechanisms seem to be highly complex, needing further elucidation before they will be explicable in terms of the metabolism of the hosts and the pathogens. The role of the polyphenoloxidases in these systems is also of considerable interest. Johnson & Schaal (1952) reported that tyrosinase converts chlorogenic acid to the quinone in damaged potato tissue, and that the latter compound may be important in resistance to *Actinomyces scabies*. It has also been suggested that a polyphenoloxidase produced by the host may play a part in the resistance of tomato plants to *Fusarium oxysporum* (Davis, Waggoner & Dimond, 1953).

The fact that *Venturia inaequalis* and *V. pirina* are not pathogenic on pear and apple, respectively, even though infection may occur, certainly does not seem to be explicable in terms of polyphenolic compounds alone. As Walker & Stahman (1955) stated, they may be subsidiary factors modifying a basic disease reaction and working in relation to other host metabolites. In that case they will, as the results suggest, be of importance in the determination of pathogenicity and varietal resistance in these two diseases. This study confirms and extends those of Wiltshire (1915), Johnstone (1931) and Rudloff (1934), each of whom suggested a biochemical basis for resistance to *Venturia* species. Nevertheless, it must be remembered that the reactions described above occurred in an abnormal environment and do not necessarily demonstrate the operation of resistance mechanisms *in vivo*. It can only be shown that cultural work is leading to relevant observations when direct evidence for the operation of similar mechanisms under natural conditions is obtained by extension of the work to the living host plants.

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REFERENCES

Bradfield, A. E. & Flood, A. E. (1949). The organic constituents of fruit plants. 1. An outline of a method of examination of the water and alcohol soluble constituents. *Rep. E. Malling Res. Sta.* p. 100.

Campbell, P. N., Work, T. S., & Mellanby, E. (1951). The isolation of a toxic substance from agenized wheat flour. *Biochem. J.* **48**, 106.

Davis, D., Waggoner, P. E. & Dimond, A. E. (1953). Conjugated phenols in the *Fusarium* wilt syndrome. *Nature, Lond.* **172**, 959.

Johnson, G. & Schaal, L. A. (1952). Relation of chlorogenic acid to scab resistance in potatoes. *Science*, **115**, 627.

Johnstone, K. H. (1931). Observations on the varietal resistance of the apple to scab (*Venturia inaequalis*, Aderh.) with special reference to its physiological aspects. Part II. *J. Pomol.* **9**, 195.

Kirkham, D. S. (1954). Significance of the ratio between the water-soluble aromatic and nitrogen constituents of apple and pear in the host-parasite relationships of *Venturia* species. *Nature, Lond.* **173**, 690.

Kirkham, D. S. (1956). A culture technique for *Venturia* spp. and a turbidimetric method for the estimation of comparative sporulation. *Nature, Lond.* **178**, 550.

Kirkham, D. S. (1957). Relationships between cultural characters and pathogenicity in *Venturia inaequalis* and *Venturia pirina*. *J. gen. Microbiol.* **16**, 360.

Leben, C. & Keitt, G. W. (1948). *Venturia inaequalis* (Cke.) Wint. V. The influence of carbon and nitrogen sources and vitamins on growth *in vitro*. *Amer. J. Bot.* **35**, 337.

Rudloff, C. F. (1934). *Venturia inaequalis* (Cooke) Aderhold. 1. Der Einfluss des Nährbodens auf den Pilz und die Erhaltung seiner Pathogenität. *Gartenbauwiss.* **9**, 65.

Siebs, E. (1955). Untersuchungen über die Schorfresistenz von Birnen. 111. Stofflicher Hinweis auf die Grundlagen der Blattschorfresistenz. *Phytopath. Z.* **23**, 37.

Walker, J. C. & Stahman, M. A. (1955). Chemical nature of disease resistance in plants. *Annu. Rev. Pl. Physiol.* **6**, 351.

Wiltshire, S. P. (1915). Infection and immunity studies on the apple and pear scab fungi (*Venturia inaequalis* and *V. pirina*). *Ann. appl. Biol.* **1**, 335.

EXPLANATION OF PLATE

Fig. 1. Chromatograms of three polyphenolic fraction extracts each of Cox (Cx), Miller (M), Williams (W) and Conference (C). Extracts 1 and 3 are of young leaves, extract 2 is of mature leaves. Identified components are numbered as follows: arbutin (1), chlorogenic acid (2), phloridzin (3), phloretin (4), *iso*chlorogenic acid (5) and hydroquinone (6).

Fig. 2. Chromatograms of stages in the decomposition of arbutin and chlorogenic acid by *Venturia inaequalis*, clones E1 and A7. The significance of the numbers 1–12 is explained in Table 10.

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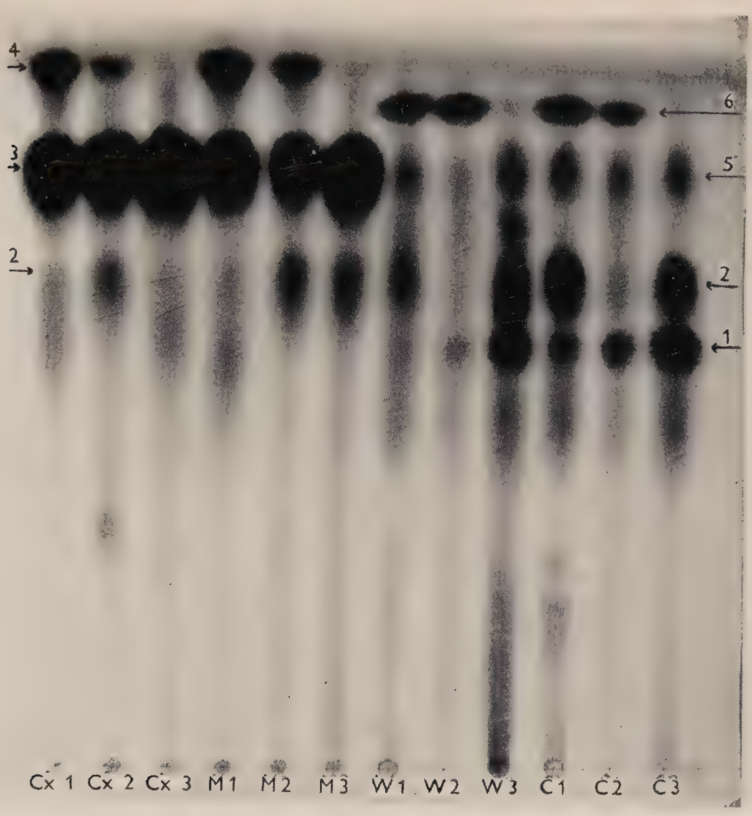
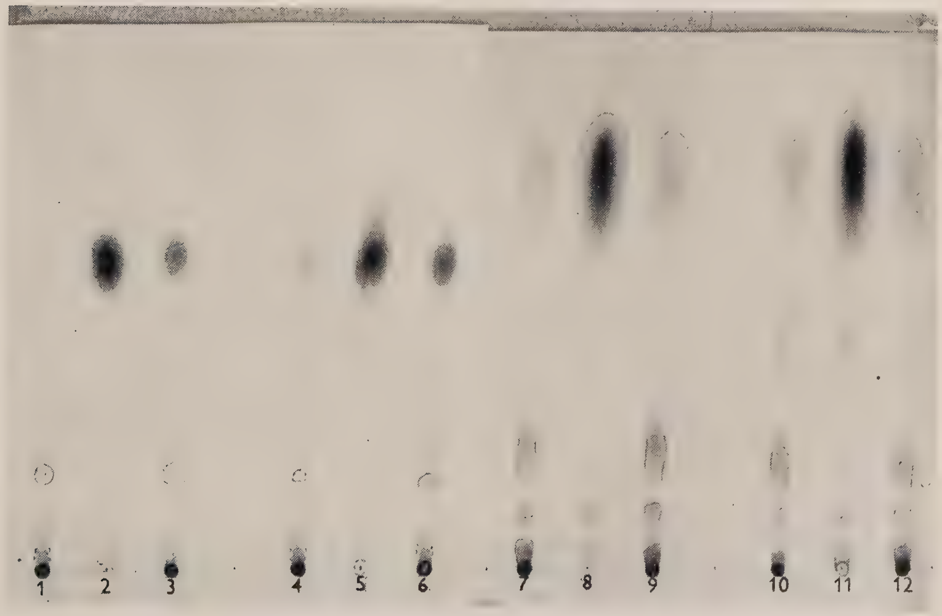

4
3
2
6
5
2
1
Cx 1
Cx 2
Cx 3
M 1
M 2
M 3
W 1
W 2
W 3
C 1
C 2
C 3

Fig. 1


1
2
3
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5
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7
8
9
10
11
12

Fig. 2

D. S. KIRKHAM—*VENTURIA* SPECIES AND HOST METABOLITES. PLATE 1

(*Facing p.* 134)

GREGORY, P. H. & HIRST, J. M. (1957). *J. gen. Microbiol.* 17, 135–152

The Summer Air-Spora at Rothamsted in 1952

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SUMMARY: The air over an arable field at Rothamsted Experimental Station, Harpenden, was sampled from 1 June to 25 October 1952 at 2 m. above ground with an automatic volumetric spore trap. Each day's slide was scanned and all the spores counted on an area representing a sample volume of 41 l. of air. Spores were classified in 20 morphological groups and a miscellaneous one. Seasonal periodicities are presented as 6-day running means of the daily average number of spores/m.^{3} air, and then related to meteorological data. Cladosporium conidia accounted for 46 % of the total catch; hyaline basidiospores (chiefly Sporobolomyces) for 31 %; and pollen only 1 %. The relative frequency of various spore types differs from that recorded by earlier workers because the suction trap catches spores of all sizes with almost equal efficiency and is little influenced by external conditions. The results which should be representative of large rural areas of central and south England show that the major changes of spore concentration depend on the weather and the phenology of the local vegetation and its associated fungal flora. During 24 days in late June and July comparable estimates of spore concentration were made with another trap 24 m. above ground. The spore concentration of the twelve commonest groups at 24 m. totalled 82 % of that at 2 m.

The sampling of air continuously over long periods for air-borne micro-organisms was first practised by Schoenauer (1876) and by Miquel (1877–99) at the Observatoire Municipal Montsouris, Paris. Some of their methods collected representative samples but others were selective. Selective methods have been used in most subsequent surveys, either intentionally (Neill & Armstrong, 1955) or because the traps used caught spores with variable efficiency (Gregory, 1950) and favoured the recognition of certain groups. Long-period surveys have usually relied on exposing sticky microscope slides or Petri dishes (Bernstein & Feinberg, 1942; Walton & Dudley, 1940).

Both slides and Petri dishes catch large spores more efficiently than small ones and the efficiency of both depends on wind speed; also dishes are exposed only for short periods and record only those organisms that grow to form macroscopic colonies. The data we present are derived from the catches by automatic volumetric spore traps, which are much less affected by changes in wind speed and which catch spores of greatly differing size with almost equal efficiency (Hirst, 1952). The catches, examined microscopically under an oil-immersion objective, are therefore thought to reveal the relative and absolute frequency of all air-borne spores larger than 3μ. more accurately than was possible until now. The method is limited by the difficulty of classifying spores visually; more specialized methods will be required for further study of many groups.

METHODS

Trapping methods

Two traps were used; one (trap A) with its orifice 2 m. above ground level, from 1 June to 25 October 1952, and the other (trap B) at a height of 24 m. above ground level from 20 June to 13 July 1952.

Trap A was that originally described by Hirst (1952). It was placed on the Garden Plots Enclosure, Great Field III, Rothamsted Experimental Farm, Harpenden (National Grid Reference TL132133), just over 400 ft. above sea level, *c.* ½ mile south and west of the built-up area of Harpenden (population 17,000) and 25 miles N.N.E. of the centre of London. The nearest open sea is about 50 miles to the east and 70 miles to the south, but the prevailing south-westerly wind has a fetch, over land, of some 120 miles after crossing the Dorset coast. The crops and vegetation of the district are fairly typical of rural conditions in the south of England. The vegetation of the area within a quarter of a mile radius of the trap is shown in Fig. 1. The trap was placed at the centre of an almost square ¼ acre plot of maincrop potatoes, primarily to study the dispersal of the sporangia of the potato blight fungus *Phytophthora infestans*. This crop and the field of barley to the south-west which became heavily infected with powdery mildew (*Erysiphe graminis*) supplied more spores of these types than was typical of the district.

Trap B was similar in all essential features, except that an oil seal was incorporated in the column which allowed completely free rotation of the wind vane and impactor assembly, without interfering with continuous suction. This modification makes the apparatus more compact, allows the orifice to be placed as little as ½ m. above ground level, and made it easier to fix it to a bracket on the outside of a lattice mast 24 m. above ground level. When so fixed the trap was 22 m. above trap A and 100 m. to the S.S.E. It was in use continuously from 20 June to 13 July 1952, except for 29 June.

Weather and phenology in the summer of 1952

Daily records of sunshine, rainfall, temperature and humidity, obtained from instruments adjacent to trap A or in the meteorological enclosure close to trap B, are presented in Fig. 2, as 6-day running means.

Conditions in spring allowed cereal crops to be drilled early but snow late in March delayed the planting of root crops. Plants grew rapidly during April and May, both warm months with sunshine and rainfall near average. Grasses came into flower during late-May and June and an average hay crop was harvested between 6 and 30 June. June and July were unusually warm and dry. Despite lack of rain, cereal crops continued to grow well; they ripened earlier than usual and harvest began on 23 July. Temperatures remained slightly above average in August, when there was nearly twice as much rain as the average, which interfered with the cereal harvest and favoured the growth of saprophytic moulds. Root crops, which had suffered from the midsummer drought, grew rapidly both in August, and in September which was a cold wet month. Potato plants, already maturing in August, were attacked by potato blight during the second half of the month and during September. The autumn flush of toadstools (Agaricaceae) began, as usual, in August but was checked by a dry spell at the end of the month. The usual peak in September was not reached, perhaps because low temperatures and damaging frosts occurred unusually early.

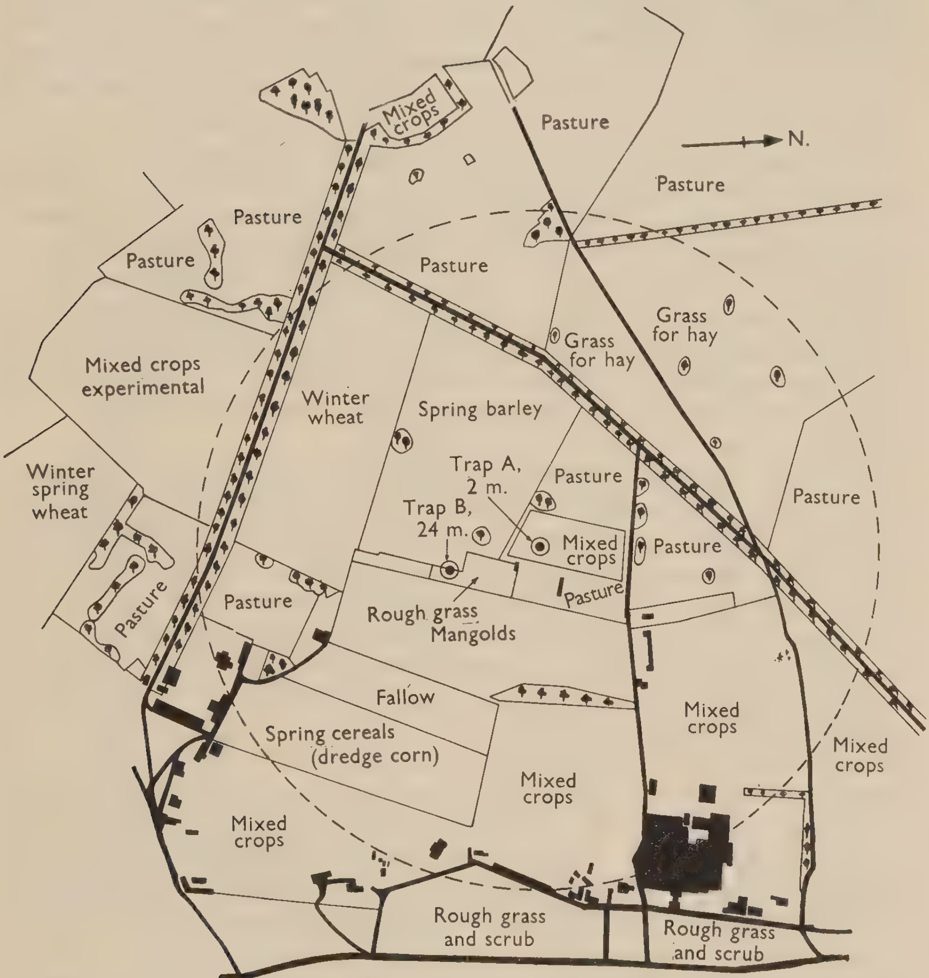
Mixed crops
Pasture
N.
Pasture
Pasture
Pasture
Pasture
Grass for hay
Grass for hay
Mixed crops experimental
Winter wheat
Spring barley
Winter spring wheat
Trap A, 2 m.
Trap B, 24 m.
Pasture
Mixed crops
Pasture
Pasture
Pasture
Pasture
Pasture
Rough grass
Mangolds
Fallow
Spring cereals (dredge corn)
Mixed crops
Mixed crops
Mixed crops
Mixed crops
Rough grass and scrub
Rough grass and scrub

Fig. 1. Map showing position of traps and vegetation in 1952. Dotted circle is a quarter mile radius from trap A.

Scanning procedure

The slides, prepared as described by Hirst (1953, p. 377), were changed daily at 09.00 hr. G.M.T., and all estimates of daily mean spore concentration apply to the 24 hr. preceding 09.00 hr. on the date quoted. As most of the data are presented as 6-day running means, the discrepancy from the daily period midnight to midnight is immaterial.

Daily mean concentrations of various types of spores were estimated from counts of spores on a single narrow traverse parallel to the direction in which the slide moved while in the trap ('long traverses', see Hirst, 1953, p. 378).

This method of scanning is relatively rapid and samples the whole of the exposure period, but it does not reveal the typical diurnal periodicities of the spore types or the brief maximum concentrations, which a cursory glance showed were many times greater than the daily mean. Each traverse was 40 μ. wide and approximately $50 \times 1000\,\mu$. long, or 2 mm.^{2} in area; pollens and uredospores were counted on traverses twice this width. A traverse 40 μ. wide corresponds to a sample air volume of 41·15 l./day. A sample of this size allows accurate estimation of only the more abundant forms. This type of trap concentrates many spores on a small area, so that little time is wasted in scanning empty areas. For instance there were on average over 120 Cladosporium conidia/mm.^{2} of slide surface throughout the whole period, and on the peak day the number rose to 770/mm.^{2}. A larger area could have been scanned in the same time had an objective of lower magnification been used, but experience shows that it is desirable to use an oil-immersion objective with a magnification of × 45 and a focal length of 3·75 mm., otherwise minute hyaline spores, such as those of Sporobolomyces and Tilletiopsis, may be missed altogether.

The counts were not corrected for variations in the efficiency of trapping, (Hirst, 1953, p. 378) which was assumed to be 100 %. The actual efficiency depends on the wind speed and the size of particle and averages about 80 %. Thus all the spore numbers quoted are underestimates of the true concentration. Counts represent the total number of spores; those joined together in clumps as one dispersable unit were counted as separate individuals. This convention makes it easier to estimate the weight of any spore type in a sample of air, but is less suitable for estimating the number of reproductive units deposited/unit area.

Classification of the spores caught could be based only on microscopic evidence. We have therefore had to use special categories, which are varyingly related to standard taxonomic categories. Inorganic particles and fragments of plants unlikely to be viable were ignored. The spore categories used are arranged, for convenience of presentation, in the order shown in Fig. 2, which is divided arbitrarily into scales based on the approximate frequency of the spores at their seasonal maximum. The categories were originally chosen after a preliminary examination of all the slides, to give the most useful and reliable grouping possible by spore morphology alone. The homogeneity of the categories varies greatly. A few are probably limited to individual species, but others are very wide and ill-defined. The categories are in general 'form groups' and were devised after comparison with many reference slides of carefully identified spores and pollens. Each category is discussed individually in the next section.

THE COMPOSITION OF THE AIR-SPORA TWO METRES ABOVE GROUND

The salient features of the seasonal changes in the composition of the air-spora 2 m. above ground are shown in Fig. 2, where the categories are divided into 4 groups each plotted on a different scale. On scale A a unit represents 20 spores/m.^{3} air; on scale B–D it represents 100, 500 and 5000 spores/m.^{3}, respectively. This not only aids graphical presentation, but also stresses the importance of the two groups in scale D.

Table 1. *Contributions of categories of spores to the whole air-spora* (*see text*)

Category	Highest daily concentration: Date	Highest daily concentration: Number/m.^{3} air	Mean for season (no./m.^{3})	% of total air-spora
D. Hyaline basidiospores	6. viii.	62,500	3919	31·22
Cladosporium	8. viii.	37,100	5837	46·51
C. Hyaline rods	16. vi.	3,510	396	3·16
Unclassified	10. viii.	3,625	1010	8·04
Erysiphe	18. vi.	5,570	210	1·67
Ustilago	18. vi.	2,850	221	1·76
Alternaria	29. viii.	2,760	150	1·20
Coloured basidiospores*	25. viii.	1,820	413	3·29
B. Tilletiopsis	15. vi.	384	71	0·56
Total pollen	11. vi.	1,184	129	1·03
Uredospores	5. viii.	384	36	0·29
Ascospores B	25. viii.	288	33	0·26
Botrytis	9. viii.	288	53	0·42
A. Ascospores A	19. viii.	240	21	0·17
Phytophtora infestans	1. ix.	144	7	0·04
Ganoderma	15. viii.	216	26	0·21
Helicospores	1. vii	144	7	0·06
Polythrincium	26. viii.	26	5	0·04
Pteridium	†	36	4	0·03
Helminthosporium	‡	48	4	0·04

* Excluding Ganoderma

† This low peak concentration occurred several times particularly in late August–September.

‡ This low peak concentration occurred several times particularly in late July.

The changing frequency of each category is shown by a 6-day running mean curve of the estimated average number of spores/m.^{3} air throughout each day. To decrease the amount of calculation and tabulation required, these values were plotted only at 3-day intervals, which smooths out short-term weather changes and makes it easier to study the effects of long spells of weather and phenological changes. Day-to-day changes in spore concentration are similarly obscured, so the highest of the daily average concentrations for each category are shown in Table 1, together with the average over the whole 150-day period (1 June to 25 October) and the percentage which each contributed to the total of the whole air-spora.

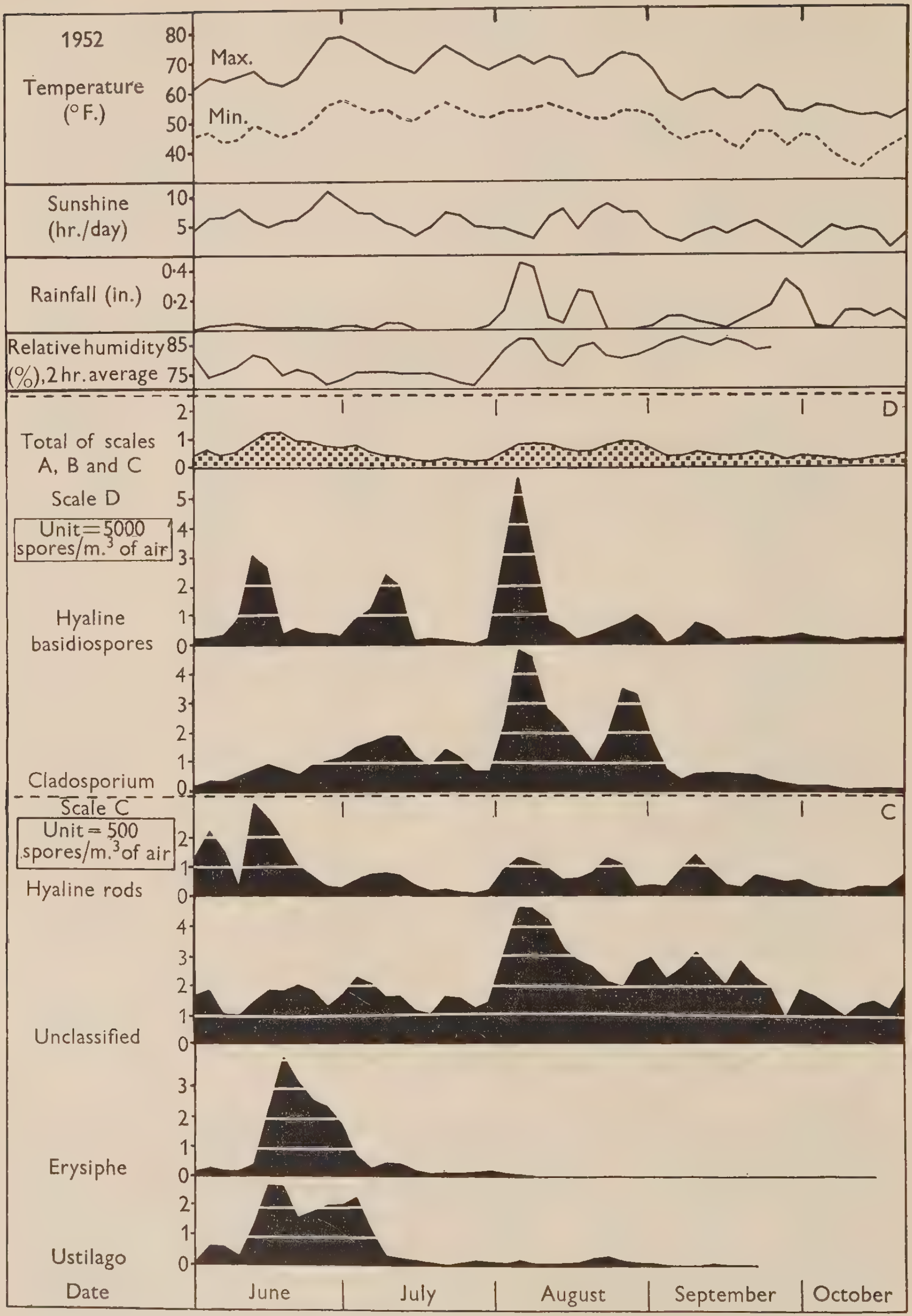

Fig. 2. Weather and seasonal periodicity of spore concentration 2 m. above ground, 1 June to 25 October, 1952. Curves are 6-day running means plotted at 3-day intervals.

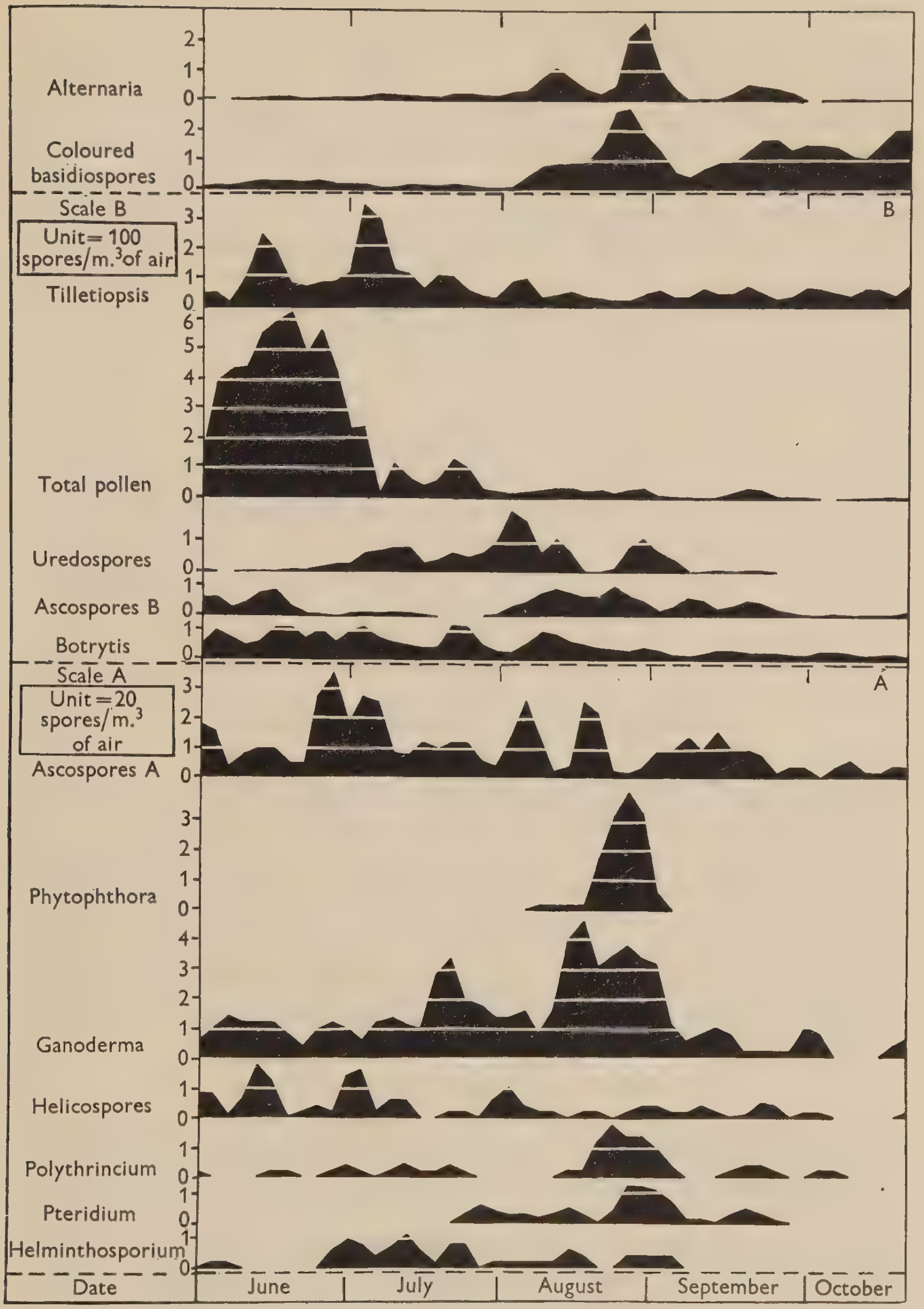

Fig. 2 (*continued*)

Helminthosporium type (*Fig.* 2, *scale A*)

Typified by the cylindrical, multi-septate, thick-walled conidia of *Helminthosporium avenae*, this distinctive group probably includes very few spores not belonging to this genus. The group featured prominently in surveys by earlier workers, but it does not in ours. Partly this is because our method of trapping is not biased in favour of catching large and heavy spores, though the difference may be partly due to the modern methods of controlling diseases of cereal crops by fungicidal seed dressings. Wild grasses or pastures probably formed the main source, but the highest concentration observed was of the same order as the 34/m.^{3} recorded by Grainger (1954) above an oat crop infected with *H. avenae* during the peak period of spore liberation. The group was commonest in dry warm weather.

Pteridium type (*Fig.* 2, *scale A*)

Typified by the spores of *Pteridium aquilinum*, the bracken fern, which are large and have a characteristic shape, colour and ornamentation, this is probably another homogeneous group. There are no large areas of bracken within several miles of the trap site but small quantities occur within one mile. These spores were caught most frequently in warm dry weather from late July to September. The sporangia of *P. aquilinum*, like those of many other ferns, depend on desiccation for dehiscence and spore ejection (Ingold, 1939).

Polythrincium trifolii *type* (*Fig.* 2, *scale A*)

This group of very characteristic spores was founded on specimens of the Polythrincium conidial state of *Cymadothea* (*Dothidella*) *trifolii*, the cause of clover blotch. All spores included probably belonged to this species, which was common on wild white clover (*Trifolium repens*) all around the trap sites. The group, separated because of its biological interest, occurred throughout the period, but never in large numbers. Hirst (1953) found that it had a diurnal periodicity suggesting that drying liberated the spores. Wet weather decreased the number in the air, but most were caught in late August after a wet period and coincident with the peak of *Phytophthora infestans*.

Helicosporium type (*Fig.* 2, *scale A*)

This group includes any hyaline conidia coiled in a flat spiral. They probably come from cereals and grasses, for spores of this type, belonging to an unidentified species of *Helicosporium*, have often been found on dead culms of wheat at Rothamsted. The group was present throughout the season and occurred during rain, which suggests that dispersal may be aided by rain splash, although this is difficult to reconcile with the similarity of Helicosporium's seasonal periodicity to that of Tilletiopsis. Most occurred during warm spells in June and July and, in common with many other groups, few spores were caught in the cold spell in October.

Ganoderma type (*Fig.* 2, *scale A*)

This group was based on reference slides of the basidiospores of *Ganoderma applanatum* (Polyporaceae) of which there are several sources in the neighbourhood. The spores are characteristic, with an outer hyaline wall penetrated by projections of the inner brown wall. They probably all belong to *G. applanatum* since the less common *G. lucidium* has smaller spores. This group was present continuously except during the cold spell in October. It was most common in August and during the dry spell in July.

Phytophthora infestans *type* (*Fig.* 2, *scale A*)

This fungus, causing late blight of potato, is another of the types which can be referred to a species with reasonable certainty. The only other downy mildews present in recognizable quantity, *Peronospora effusa* and *P. parasitica* were placed in the Unclassified group. *P. infestans* sporangia can be distinguished from these by their size, shape, colour and the character of the papilla and stalk. The potato crop in which the trap was placed was an abundant source and the numbers recorded are therefore atypical of the district as a whole.

Ascospores type A (*filiform type, see Hirst*, 1953; *Fig.* 2, *scale A*)

Most of the obvious ascospores were placed in the 'Unclassified' group, for want of features that allowed definite identification, but two very characteristic although arbitrary form groups were recorded separately. Type A consisted of long filiform spores resembling reference slides of ascospores of *Epichloe typhina*, but no doubt including spores from diverse genera such as *Claviceps*, *Geoglossum*, *Cordyceps*, etc. Some filiform conidia of Fungi Imperfecti may have been included, but they probably form only an insignificant part of the group. Ascospores type A occurred typically in damp air during rain but they were also to be found with high humidity at night. Dews probably caused some liberation and may explain the considerable numbers caught during the dry spell in July. (The second group, Ascospores type B, is described below, after *Botrytis*).

Botrytis type (*Fig.* 2, *scale B*)

Spores of this type resemble those of *Botrytis cinerea* and form a characteristic group defined by their shape, faint colour, and the way in which they shrivelled slightly in the mountant we used. There were numerous local sources of this nearly ubiquitous fungus, on withered petals and moribund plants, as well as sources of specialized species that cause diseases of vegetables and ornamentals. They were present throughout the season, but were most common in the earlier part. Day-to-day changes were small, but the spores were more common in the air in the daytime than at night.

Ascospores type B (*Fusiform type, see Hirst*, 1953; *Fig.* 2, *scale B*)

The other distinctive type of ascospore separated was of fusiform multiseptate spores, with one of the intermediate cells distinctly swollen. These spores were probably derived from saprophytic pyrenomycetes such as Leptosphaeria. They also are characteristic of damp air but their seasonal periodicity differed considerably from that of the filiform spores included in type A, perhaps because their spores need wetter conditions for liberation. Their concentration increased with humidity, particularly when other factors indicate that the air close to the ground remained wet. They did not show the July peak which is a feature of group A.

Uredospore type (*Fig.* 2, *scale B*)

This category included all spores which could be recognized unquestionably as uredospores of rust fungi (Uredineae). Uredospores of less common species may have been placed in the Unclassified group. Wheat, barley and grasses no doubt formed the main source. Crops of wheat within a few hundreds yards of the trap were infected with *Puccinia glumarum*, *P. triticina* and *P. graminis* and a nearby barley crop with *P. anomala*. Rust diseases of cereals were more than usually common in 1952, uredospores were nevertheless only a minor component of the air-spora. Most uredospores were caught in warm spells, particularly in August, and the numbers declined rapidly after mid-September, presumably because of harvesting.

Total pollens (*Fig.* 2, *scale B*)

Three pollen categories were used: 'winged' (mostly *Pinus*), 'grass' and 'others'. The last was mainly weeds, including Chenopodiaceae and *Urtica*. Sampling was started after the season of pollen liberation by deciduous trees, so few of these are included in the miscellaneous group. As the incidence of pollens has been studied elsewhere (Hyde, 1952) only the concentration of total pollens is given in Fig. 2. The grass pollen group was predominant, particularly during the peak period in June. The seasonal periodicity of the total pollen group is governed chiefly by the flowering of grasses, and release, which is favoured by warm sunny weather, is greatest in the daytime (Hyde & Williams, 1945, 1946; Hirst 1953).

Tilletiopsis type (*Fig.* 2, *scale B*)

This type consists of minute basidiospores often folded hinge-like into unequal parts. The group is so distinctive that it was counted separately before it was shown that the spores were identical with the basidiospores (ballistospores) of Tilletiopsis. Last (1955*a*) showed that this genus, a member of the Sporobolomycetaceae and close relative of the pink 'mirror yeasts', is a common epiphytic fungus on the leaves of cereals in summer. It is interesting to note that it was most common in June and July concurrently with peak numbers of 'Helicospores', during spells with warm nights and moderately high average relative humidity.

Coloured basidiospore type (*Fig.* 2, *scale C*)

The basidiospores produced by many fungi belonging to the Agariacaceae and related families are characterized by a distinct apiculus and often a germ pore, firm and often thin walled, with pure and intense colours. Examination of many reference slides of basidiospores, identified by specialists in this group of fungi, showed that many were sufficiently distinctive to be recognizable as species, and the whole form a readily recognizable group. Spores of *Coprinus* spp., *Boletus* spp., *Hypholoma* spp., etc., were often seen. *Psilocybe foenisecii* and *Bolbitius vitellinus* were recognized with reasonable certainty as species. The occasional spores of *Coniophora* spp., were placed in the Unclassified group until their identity was recognized. As would be expected, most appeared in August and September (see Gregory & Hirst, 1952) and our data suggest that peak numbers occurred when warm spells came about 2 weeks after periods with substantial rain. Taxonomically the *Ganoderma* type should of course, be included with the coloured basidiospores.

Alternaria type (*Fig.* 2, *scale C*)

This group was widely interpreted to include dark muriform conidia differing from one another as much as the rounded spores of *Stemphylium botryosum* differ from the club-shaped conidia of *Alternaria tenuis*, which was by far the most common genus included. The group, more important as saprophytes than parasites in this country, was most common during August particularly in warm dry periods following wet spells, and *Alternaria* was the dominant genus.

Ustilago type (*Fig.* 2, *scale* C)

This category, unlike the last, was narrowly interpreted and included only spores morphologically indistinguishable from *Ustilago avenae* (including *U. perennans*). Spores of most other smuts would have been placed in the Unclassified type, either because of the difficulty of distinguishing them from Fungi Imperfecti such as *Periconia* spp., or because, like Tilletia and Urocystis, they were uncommon. Spores in the Ustilago category may well have come considerable distances, but there were local sources both on cereals and on *Arrhenatherum elatius*, a common weed grass. The seasonal periodicity of the group is clearly determined by its exposure on infected grass inflorescences in June and early July. Few occur later, even during harvesting.

Erysiphe type (*Fig.* 2, *scale C*)

This group comprised conidia (*Oidium* state) derived from powdery mildews. *Erysiphe graminis* probably predominated, but *E. polygoni* was also seen and the conidia of other genera may have made a small contribution.

The group was present throughout the season, although in the latter part they were too few for accurate representation on the scale used. Cereal crops were probably the most important source, and Last (1954, 1955*b*) showed that late-sowing rendered crops susceptible. A late-sown crop of spring barley to the south-west of the trap was heavily attacked in June, and this important local source doubtless dominated others in determining the seasonal periodicity found for this group.

Unclassified types (Fig. 2 scale C)

The composition of this group changed considerably throughout the season; spores were included in it for the following reasons:

(1) Spores that could have been referred to 'generic types', but which were often too rare to form quantitatively significant components of the air-spora. These included spores of the genera *Tilletia, Urocystis, Epicoccum, Tetracladium, Lycoperdon, Fusarium* (very rare), *Sordaria, Chaetomium, Peronospora, Torula, Penicillium and Aspergillus.* Some of these have been found abundantly when sampling special environments, e.g. *Penicillium* and *Chaetomium* in a mushroom house.

(2) Spores which could be less precisely recognized, such as the conidia of the Entomophthoraceae, spore types of the rusts other than the uredospores, the soredia of lichens, spores of bryophytes and pteridophytes other than Pteridium.

(3) Spores not classifiable because of unsuitable orientation on the adhesive surface or because they were partially obscured by debris or other particles.

(4) Spores, perhaps distinctive, but unclassifiable because of ignorance on the part of the recorders.

The Unclassified category is needed to fulfil the object of the survey, which was to make a total count of spores, and not only to group the forms occurring most abundantly into named taxonomic categories. Such a heterogenous group could hardly be expected to show any clear relationships between numbers and the weather. The fact that the group was most common during wet weather in August merely emphasizes how little is known of the many featureless components of the damp air-spora.

'Hyaline rods' type (Fig. 2, scale C)

This was an abundant and distinctive group to the identity of which we have few clues. The spores were about $3–5 \times 0{\cdot}7–1{\cdot}0\,\mu$. with somewhat rounded ends, straight or slightly curved and the wall refractile like that of a fungus spore. Sometimes a minute granule inside the cell appeared to touch the end wall. Many spores were distinctly biguttulate and resemble spores of Phoma or Phyllosticta type, but they were smaller than most species known in Britain. They might be the microconidia ('spermatia') of a pyrenomycete such as one of the Mycosphaerellaceae. They were small for ascospores and seldom occurred in groups on the trace. The seasonal periodicity of the group resembles that of other members of the damp air-spora already described, e.g. Helicospores, Tilletiopsis, and ascospores A and B.

Cladosporium *type* (*Fig.* 2, *scale D*)

Reference slides showed that the spores from a single specimen or culture of *C. herbarum* varied greatly in size, colour and roughness of the surface. Nevertheless, the scanner soon learns to identify members of this category with confidence, especially after encountering the digitate clusters of conidia often found on the slides on windy days, which contain all stages of development. *C. herbarum* formed the majority of the catch but *C. macrospora* (Richards, 1953) was recognizable and other species were no doubt included. This group was a dominant constituent of the daytime air-spora, particularly in rural districts. It was present in considerable numbers from June to October, with maximum concentrations in August. The number in the air changes very rapidly with changing weather (Hirst, 1953; Gregory, 1954); spores are produced abundantly in high temperatures and high humidity. The seasonal maximum probably depends on the accumulation of dead plant tissues among grasses, cereal and root crops, during the late summer when conditions favour spore production.

Hyaline basidiospores (*Fig.* 2, *scale D*)

It would have been desirable to subdivide this category, but this was not realized when scanning was started. One subgroup would contain the hyaline basidiospores of the Agaricales, whose spores show a periodicity similar to those in the coloured basidiospore group. The second subgroup, vastly more abundant and typically predominant late at night and in the early morning, and by day during damp weather, has much smaller spores and is now recognized as belonging to the genus *Sporobolomyces*. This connexion has been confirmed by direct examination of the sporing colonies on cereal leaves by Last (1955*a*) and by drawing air through filter-paper, which was later incubated on nutrient medium to develop the pink colonies of Sporobolomyces.

The highest concentrations of this group occur in the early hours of mornings of low turbulence accompanied by long periods of warm humid nights. The category is second only to Cladosporium in average concentration over the whole season and actually achieved the highest daily mean concentration (Table 1).

RESULTS TWENTY-FOUR METRES ABOVE GROUND

Information about changes in spore concentration at different heights above ground would help to answer many questions concerning the distances to which spores are transported and the rate at which they are deposited. Grainger (1954) and Last (1955*c*) reported steep gradients of spore concentration in and just above crops. Others, mostly in North America, have caught spores from aircraft flying at considerable altitudes. Several long series of trap exposures have been made on the roofs of buildings, chiefly by people studying allergy, but there are few records of simultaneous spore trapping at different heights. Part of the present series compares spore concentration 2 m. above ground and at a well-exposed site high in the zone of frictional turbulence, 24 m. above ground. The data quoted are limited to the twelve commonest groups on 24 of the days between 20 June and 14 July. The traps were separated by a horizontal distance of 100 yd. and differed in their exposure to certain local spore sources, notably a spring-sown barley crop to the south-west which was heavily attacked by *Erysiphe graminis*. The average spore concentration of the twelve groups selected measured at 24 m. was 81·5 % of that measured at 2 m. The percentage distribution among the groups varied considerably for individual groups as shown in Table 2, which also shows the number of days when trap B caught the same number or more spores than trap A. The spore concentration at the higher trap was unexpectedly large, particularly so with spore types characteristic of the night air-spora usually most common when turbulence and convection are slight. Twice as many coloured basidiospores were caught by the higher trap. This difference is, however, largely accounted for by 2 days when the concentration at trap B was 8 and 10 times that at trap A; these were the 2 days when the spore concentrations were largest. A detailed analysis of the comparison is unlikely to be profitable, for previous work close to the ground has shown the necessity for hourly counts and weather records if the factors governing spore concentration are to be understood.

Table 2. *Comparison of catches from trap A* (2 *m. above ground level*) *and trap B* (24 *m. above ground level*); 20 *June*–14 *July* (24 *days*) 1952

Category	'B' as % of 'A'	No. of days out of 24 when 'B' equalled or was greater than 'A'
Grass pollens	71	11
Other pollens	68	12
Cladosporium	90	7
Ustilago	86	9
Coloured basidiospores	204	13
Hyaline basidiospores	66	11
Alternaria	114	16
Erysiphe	37	2
Botrytis	91	14
Tilletiopsis	92	14
Hyaline rods	110	16
Unclassified	97	11

DISCUSSION

No reference has been made in these results to one important group of air-borne micro-organisms, the bacteria. Individual bacteria are particles smaller than the trap was designed to catch. They were seldom noticed while scanning unstained preparations (staining prevents the use of natural spore colour, a valuable aid to the recognition of many fungus spores). In outdoor air, bacteria are usually dispersed on 'rafts' composed of soil particles or vegetable debris, which prevent their being seen during scanning. Special methods exist for sampling air-borne bacteria (Bourdillon, Lidwell & Lovelock, 1948).

Where these have been used out of doors in summer, they suggest that fungus spores are present in greater number than bacteria. It is interesting to note that the average total microbial count of 50 units/m.^{3} recorded by Bourdillon *et al.* (1948) is strikingly low compared with our results. This may have been due to a low efficiency with which the larger particles were collected by the apparatus then used; probably 25 units/m.^{3} represents a typical concentration of the bacteria capable of growing on blood agar. This is near the detectable threshold concentration by the methods used in the survey (i.e. the number which must be present before one microbe *should* appear in the area scanned). Hence, few bacteria should have been seen except at peak times.

Mycologists and students of allergy will note with surprise that *Penicillium* and *Aspergillus* spp. have not been given separate categories. Although readily recognizable when in chains, the individual conidia from these fungi have few characteristic features and were therefore placed in the 'Unclassified' category. We have no doubt that the method used here tends to underestimate these genera. The available evidence, however, shows that *Penicillium* spp. form only a very small proportion of the spores in outdoor country air (Richards, 1954*a*; 1956). High numbers, which occur chiefly in winter, are associated with the air of special environments; indoors, particularly associated with food and in glasshouses and in cities (Richards, 1954*b*; Maunsell, 1954).

The most significant additions to the air-spora indicated by this survey are the important autumnal contribution from basidiomycetes and the enormous abundance of Sporobolomyces. Numerically Sporobolomyces rivals Cladosporium, and the related category Tilletiopsis is by no means negligible. Both Sporobolomyces and Tilletiopsis were unknown until 1930. Though predominating by night, Sporobolomyces may persist in great numbers when the humidity remains high during the following day.

Most ascospores were placed in the 'Unclassified group'. This may disguise their importance, particularly in wet weather. Studies of *Venturia inaequalis*, the fungus which causes apple scab, have shown that in orchards in early spring this one species may be an important, and at times the dominant, constituent of the air-spora (Hirst, Storey, Ward & Wilcox 1955). Similarly, little prominence was given to splash-dispersed spores. These are common among both saprophytic and parasitic fungi, but as they are often relatively featureless they are difficult to classify visually. It is possible that spores suspended in large splash droplets may not reach or be able to traverse the slit orifice of the trap unless the diameter of the droplet has been so decreased by evaporation that it has a terminal velocity similar to that of the larger spores. Workers using culture methods have recorded a small proportion of Phoma colonies (Richards, 1956). However, colonies developing on culture media may have arisen from an ascospore or from a splash-dispersed conidium, although in culture they may produce only one type of spore, or neither. Richards's Phoma component may therefore be represented either as part of our 'Hyaline rods' group or as ascospores included in the 'Unclassified group'.

The seasonal periodicities differ greatly in character, no doubt because of complex interaction of physical and biological factors. Some groups are present almost continuously throughout the period studied, with only slight changes in concentration, for example Botrytis, the helicospores and the hyaline rods. Others display pronounced peaks, such as those of pollens Erysiphe, Ustilago and Phytophthora. Non-selective sampling suggests some surprising differences in the spore productivity of various species. None of the sporophores of Ganoderma contributing to the catches were close to the trap and their total surface area must have been minute compared with that occupied by sporing lesions of *Phytophthora infestans* on the quarter acre of potatoes at the centre of which the trap was placed. Yet Ganoderma provided both higher maximum and total catches than Phytophthora over a longer period and in a greater variety of weather. Almost the only characteristic shared by all the spores studied is that of being visible under the compound microscope. A method which will collect continuously all spores with little partiality for shape or size, and which is relatively little influenced by changes in external conditions, may well be claimed useful for making general surveys of atmospheric spore content. No existing method is, however, perfect. The present one has the advantage of completeness and representative sampling, but it cannot distinguish live spores from dead ones, and only a limited number of spores is certainly identifiable. The importance of these factors depends much on the purpose for which the information is collected.

The constitution of the air-spora is studied chiefly because of its importance in studying respiratory allergy or diseases of plants. For studies of allergy the count of total numbers of spores which we have made may be very satisfactory, because it includes pollens, obligate parasites, dead spores and others which will not grow or sporulate in culture. It also allows fairly easy calculation of the total mass of spores inhaled by patients. The suction rate of 10 l./min. approximates to the volume inhaled by an adult engaged in gentle activity. The results show that a very few types are of predominant importance numerically; fortunately this allows a large proportion to be classified 'generically', with reasonable accuracy. The method fails to give adequate records of a few important known allergens, for reasons which have already been discussed.

Plant pathologists need to know the large changes which occur in the whole air-spora, but they will naturally be interested more in the behaviour of individual organisms or groups of closely related species. Many important plant-pathogenic fungi have spores which are quite characteristic; many others cannot be recognized visually with any certainty. Further study will require the development of specialized techniques for identifying and estimating the number of effective reproductive units deposited on sites where they can cause disease.

These observations were made in an area devoted to mixed farming, with land-use typical of large areas of the Midlands and the south and east of England. Local sources are known to have affected the catches to some extent; for instance, the effects of individual crops are discernible. Almost all the spores, except from Agaricaceae, were of types found on vegetable matter dead or alive. There is no evidence, at Rothamsted, to support the claims by Pady

& Kapica (1953) that soil micro-organisms contribute a significant proportion of the air-spora. The composition of the air-spora would no doubt have been different had the traps been situated on a moor or in woodland. The major changes of spore concentration seem to depend on the weather and phenology of the local vegetation and its associated fungal flora.

REFERENCES

Bernstein, T. B. & Feinberg, S. M. (1942). Air-borne fungus spores. A five-year survey of daily mold spore content of Chicago air. *J. Allergy*, **13**, 231.

Bourdillon, R. B., Lidwell, O. M. & Lovelock, J. E. (1948). Studies in air hygiene. *Spec. Rep. Ser. med Res. Coun., Lond.* no. 262, p. 356.

Grainger, J. (1954). Spore production by *Helminthosporium avenae*. *Trans. Brit. mycol. Soc.* **37**, 412.

Gregory, P. H. (1950). Deposition of air-borne particles on trap surfaces. *Nature, Lond.* **166**, 487.

Gregory, P. H. (1954). The construction and use of a portable volumetric spore trap. *Trans. Brit mycol. Soc.* **37**, 390.

Gregory, P. H. & Hirst, J. M. (1952). Possible role of basidiospores as air-borne allergens. *Nature, Lond.* **170**, 414.

Hirst, J. M. (1952). An automatic volumetric spore trap. *Ann. appl. Biol.* **39**, 257.

Hirst, J. M. (1953). Changes in atmospheric spore content: diurnal periodicity and the effects of weather. *Trans. Brit. mycol. Soc.* **36**, 375.

Hirst, J. M., Storey, I. F., Ward, W. C. & Wilcox, H. J. (1955). The origin of apple scab epidemics in the Wisbech area in 1953 and 1954. *Plant Path.* **4**, 91.

Hyde, H. A. (1952). Studies in atmospheric pollen. V. A daily census of pollen at Cardiff for the six years 1943–8. *New Phytol.* **51**, 281.

Hyde, H. A. & Williams, D. A. (1945). Studies in atmospheric pollen. II. Diurnal variation in the incidence of grass pollen. *New Phytol.* **44**, 83.

Hyde, H. A. & Williams, D. A. (1946). Studies in atmospheric pollen. III. Pollen production and pollen incidence in Ribwort Plantation (*Plantago lanceolata* L.). *New Phytol.* **45**, 271.

Ingold, C. T. (1939). *Spore Discharge in Land Plants.* Oxford University Press.

Last, F. T. (1954). The effect of time of application of nitrogenous fertilizer on powdery mildew of winter wheat. *Ann. appl. Biol.* **41**, 381.

Last, F. T. (1955*a*). Seasonal incidence of Sporobolomyces on cereal leaves. *Trans. Brit. mycol. Soc.* **38**, 221.

Last, F. T. (1955*b*). Effect of powdery mildew on the yield of spring-sown barley. *Plant. Path.* **4**, 22.

Last, F. T. (1955*c*). The spore content of air within and above mildew-infected cereal crops. *Trans. Brit. mycol. Soc.* **38**, 453.

Maunsell, K. (1954). Concentrations of air-borne spores in dwellings under normal conditions and under repair. *Int. Arch. Allergy, N.Y.* **5**, 373.

Miquel, P. (1877–1899). *Annu. Obs. Montsouris.*

Neill, J. C. & Armstrong, C. S. (1955). An aerial survey of ascospore distribution of blind-seed disease of ryegrass (*Gloetinia* (*Phialea*) *temulenta*). *N.Z. J. Sci. Tech.* **37**, 106.

Pady, S. M. & Kapica, L. (1953). Air-borne fungi in the arctic and other parts of Canada. *Canad. J. Bot.* **31**, 309.

Richards, M. (1953). Saprophytic *Cladosporium* in Britain. *Nature, Lond.* **171**, 615.

Richards, M. (1954*a*). Seasonal periodicity in atmospheric mould spore concentrations. *Acta allerg., Kbh.* **7**, 357.

Richards, M. (1954*b*). Atmospheric mould spores in and out of doors. *J. Allergy.* **25**, 429.

Richards, M. (1956). A census of mould spores in the air over Britain in 1952. *Trans. Brit. mycol. Soc.* **39**, 431.

Schoenauer, M. (1876). *Annu. Obs. Montsouris.*

Walton, C. H. A. & Dudley, M. G. (1940). Pollen survey in Manitoba. *Canad. med. Ass. J.* **42**, 430.

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HALLIWELL, G. (1957). *J. gen. Microbiol.* **17**, 153–165

Cellulolysis by Rumen Micro-organisms

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SUMMARY: The optimal conditions for accurate and reproducible analysis of cellulose hydrolysis by micro-organisms, particularly from the rumen, were determined. Rumen micro-organisms were found to require approximately 50 mg. substrate (cellulose powder) in a 40 hr. incubation at 37° with 1 ml. of rumen liquor, and a total volume of 8 ml., in static vessels. All types of cellulose or simpler substituted derivatives, from soluble carboxymethylcellulose through insoluble cellulose powder to cotton fibres, are nearly quantitatively hydrolysed by mixed rumen micro-organisms. The effect of the inorganic composition of the medium, of antibacterial agents (thymol, fluoride, toluene) and of concentration procedures on the cellulolytic activity of rumen micro-organisms was examined. Rumen protozoa appear to play a minor role in cellulose degradation; their effective activity is due mainly to closely associated, possibly ingested, bacteria. The validity of the results obtained by various methods of 'cellulase' assay is discussed.

Much of the earlier work on cellulose metabolism in the rumen has been devoted to examination of the final products of digestion, with particular emphasis on the fatty acids formed (for reviews see Phillipson, 1953; Phillipson & Cuthbertson, 1956). In order to investigate the mechanism of action of cellulose-decomposing enzymes a new approach seems to be required, and in the work described below emphasis is placed on a less well-known stage of cellulose metabolism, namely the primary degradation of cellulose. This stage was chosen because all subsequent stages, including production of many and varied products, depend on it. Although some micro-organisms can readily solubilize the most complex forms of cellulose no one has yet succeeded in obtaining cell-free extracts having this property. On the other hand, many workers have prepared extracts (both from cellulolytic and non-cellulolytic organisms) which rapidly attack soluble cellulose derivatives, but which have little or no effect on insoluble forms of cellulose; the review by Siu (1951) provides many references to such examples. More recent examples have been collected in the list which follows and the percentage figure quoted for hydrolysis has been calculated (where possible) as a fraction of the initial amount of cellulose powder or cotton. Such 'cellulase' preparations have been obtained from fungi (Jermyn, 1952, 0·2 %; Nishizawa & Kobayashi, 1953; Whitaker, 1953, 2 %; Aitken, Eddy, Ingram & Weurman, 1956, 6 %; Basu & Pal, 1956, 4 %; Higa, O'Neill & Jennison, 1956; Thomas, 1956, 5 %), from rumen bacteria (Halliwell, 1954, unpublished, 10 %; Kitts & Underkofler, 1954, 3 %) and from animals such as the limpet, *Patella vulgata* (Festenstein & Halliwell, 1955, 4 %, unpublished). Walseth (1952) appears to be the only worker to have obtained appreciable action (60 % hydrolysis in 40 hr.) on an insoluble, though slightly degraded, cellulose. The degree of hydrolysis depended, however, on the extent of treatment with concentrated phosphoric acid. Walseth's extract was obtained from a commercial supply of *Aspergillus niger* which, however, is no longer available.

In the field of rumen cellulolysis the problem is further complicated by the necessity for anaerobiosis. Attempts made to isolate cellulolytic bacteria from the rumen have invariably used degraded or hydrolysed forms of cellulose such as the acid-treated (11 N-HCl) and ball-milled cotton of Hungate (1950*a*, *b*) or the ball-milled filter-paper of Bryant & Burkey (1953). Sijpesteijn (1951) similarly isolated Gram-positive streptococci which showed poor cellulolytic growth in pure culture on filter-paper. Hungate (1950*a*) summarized the work on cellulolytic micro-organisms by stating 'the low rate at which (chemically treated) cotton and filter-paper is digested in cultures does not imply that development in the rumen is thus limited'. However, it is clear that pure cultures of rumen micro-organisms show much less true cellulolytic efficacy as compared with the activity of mixed rumen micro-organisms.

It was evident that an investigation was required to determine the conditions necessary for the optimal rate of cellulolysis and of cellulase production by the rumen microflora. This has been attempted in the present work by using mixed cultures, which seem preferable for at least two reasons: (1) mixed cultures provide less divergence from *in vivo* conditions, perhaps because mixed enzymes from different organisms are required for cellulolysis; (2) our mixed cultures, unlike the pure strains used by other workers, act relatively rapidly and completely on all forms of insoluble cellulose tested. Finally, cellulose degradation has been determined by gravimetric analysis of residual cellulose; determination of the reducing sugars produced by rumen micro-organisms in presence of antiseptics is not applicable, for such inhibitors decrease true cellulolytic activity as opposed to the action on soluble cellulose derivatives.

METHODS

Several different forms of cellulose were used:

Native cotton fibres (Peruvian Tanguis, kindly supplied by the British Cotton Industry Research Association) were used untreated, and also de-waxed as follows. Fibres were purified by Soxhlet extraction with redistilled ethanol for 8 hr. and with ether for 6 hr., followed by refluxing under nitrogen in 1 % (w/v) sodium hydroxide for 8 hr. After washing with boiled water until neutral, washing was continued successively with cold water, 1 % (v/v) acetic acid, and water. The product was finally dried in air. *Cellulose powder*, referred to in the text as cellulose powder (Whatman), was standard grade ashless powder for chromatography and was used untreated. *Swollen cellulose* was prepared from absorbent cotton and also from cellulose powder (Whatman) by soaking in phosphoric acid (A.R., 90 %) at 1° for 2 hr., followed by washing until acid and phosphate free (Walseth, 1952). *Hydrocellulose* was prepared from absorbent (de-waxed) cotton by immersion in 75 vol. of 11 N-HCl. (A.R.) for 48 hr. at 18°. After rejecting the supernatant fluid the fine powder was poured into water and then filtered off on a Buchner funnel, washed acid- and chloride-free and finally dried in vacuum at room temperature. A product similar to this has been widely used in work on cellulolytic micro-organisms (Hungate, 1942, 1950*a*, *b*; Sugden, 1953), after further degradative treatment by grinding.

Sources of cellulolytic enzyme

A. Rumen liquor. The animals used were Cheviot sheep fitted with a permanent rumen fistula. Daily diet comprised 500 *g* hay and 250 *g* grass cubes at 7 a.m. and at 4 p.m. with free access to water and salt lick. Rumen liquor (RL; not more than 1·5 l. in 24 hr. from one animal) was filtered through six layers of surgical gauze before use.

B. Rumen liquor supernatant fluid (R.L.S.) and rumen liquor concentrates (R.P.). R.L.S. was obtained by centrifugation of filtered rumen liquor at 20,000 *g* for 30 min. at 1°. The clear brown supernatant fluid (R.L.S.) so obtained was entirely lacking in activity against all the forms of insoluble cellulose described above. The precipitated organisms from this centrifugation are designated R.P. in the sequel.

Determination of the cellulolytic activity of rumen micro-organisms

The standard assay for cellulolytic activity. Fifty mg. of insoluble cellulose (or 30 mg. with some forms of cellulose) was added to a CO_2-saturated, $NaHCO_3$ + salts buffer (see below), with or without addition of rumen micro-organisms (1 ml.). The assay tubes, 150 mm. × 15 mm., were closed with a Bunsen valve. The total volume was made up to 8 ml. (initial pH value 6·8) and the tubes were incubated at 37° for about 40 hr. Unless stated to the contrary, $NaHCO_3$ buffer or medium had the following composition: 0·2 M-$NaHCO_3$, 100 ml.; 0·154 M-KCl, 4 ml.; 0·154 M-KH_2PO_4, 1 ml.; 0·154 M-$MgSO_4$, 1 ml.; 0·154 M-$(NH_4)_2HPO_4$, 5 ml.; 0·11 M-$CaCl_2$, 3 ml. (Elsden, 1945).

Method of analysis of cellulose breakdown. Breakdown of insoluble cellulose was followed by gravimetric determination of the residual cellulose. Sintered glass filters (grade 3) were used, on which the residual cellulose was washed successively with 5 ml. volumes of 3·8 N-HCl, 0·7 N-NH_4OH, 1 % (w/v) Teepol XL, and distilled ethanol (10 ml.), with a 20 ml. water-wash between each solvent. The residual cellulose was subsequently dried at 100° for 16 hr. The author is indebted to Dr G. A. Levvy for details of this procedure. Control determinations with tubes containing insoluble cellulose and all reagents except micro-organisms did not show any loss of cellulose in periods up to 7 days at 37°, or any sign of microbial growth when examined in films stained by Gram's method. Results were corrected, where necessary for apparent endogenous cellulose of 'cellulase' preparations. After the standard solvent washing of cellulose + rumen liquor (R.L.) or precipitated organisms (R.P.), the endogenous cellulose of the microbial preparation was negligible in the case of rumen liquor and less than 4 % of the weight of cellulose used in the assay, when using precipitated organisms (R.P.). In the text, the degradation of cellulose is given in terms of '% hydrolysis' (cellulolysis). This indicates the fraction of the initial cellulose which had been solubilized.

RESULTS

Properties of rumen liquor and of rumen liquor organisms

The conditions necessary to provide a uniform response in the determination of the cellulolytic activity of various preparations are illustrated in Figs. 1–3. It can be seen that maximal conditions for cellulose loss were met by using 50 mg. cellulose powder (Whatman; C.P.W.), 40 hr. incubation at 37° and 1 ml. of rumen liquor in a total volume of 8 ml., in static vessels. Fig. 2 illustrates the importance of maintaining contact between the micro-organisms and the insoluble substrate for cellulolysis to occur. Shaking by itself (Warburg bath, 120 strokes/min., horizontal movement about 50 mm.) apparently had no deleterious effect on the integrity of the rumen liquor organisms. This was shown by stopping the agitation, whereupon cellulolysis began again at the normal rate. Similar results were obtained when stoppered vessels were substituted for those equipped with Bunsen valves. This phenomenon was also seen on passing carbon dioxide through the tubes at a rate (2 ml. CO_2/min.) sufficient to keep the cellulose + bacteria in suspension; when the flow of gas was stopped cellulose hydrolysis began again in the usual way.

Justification of the buffer medium used in the 'cellulase' assay appears in Table 1. Of the five salts present in the bicarbonate + CO_2 buffer, only $MgSO_4$ and $(NH_4)_2HPO_4$ were essential for cellulose decomposition. No improvement was found on varying the concentration of the other salts, nor was it possible to replace the bicarbonate by a $KH_2PO_4 + NaOH$ buffer of the same pH value. Addition of a reducing agent (Na_2S, 0·03 %, w/v) was not essential for optimal cellulolysis by rumen liquor nor by concentrates of the micro-organisms from the liquor (R.P.), provided that the bicarbonate + cellulose buffer mixture was thoroughly gassed with CO_2 before and after addition of organisms. However, it was advisable to maintain anaerobiosis, because the system was extremely sensitive to oxidation, as shown by the completely inhibitory effect of ferricyanide. Although 'cellulase' was never detected in the rumen liquor surrounding the bacteria it was felt that this might be due to the labile nature of the enzyme system. In an attempt to stabilize possible free cellulolytic enzymes dilute gelatin was used as an addition to the standard assay medium. It did not, however, produce the desired stimulatory effect. The centrifuged supernatant fluid from rumen liquor (R.L.S.), which is itself inactive on cellulose, did not improve or decrease cellulolysis by rumen liquor or by the deposited organisms. Cellulolysis by the deposited organisms was as effective at pH 6·6 as at the standard assay value of pH 6·8, but a further decrease of pH value to 5·9 caused complete loss of cellulolytic activity. Although the above determinations were carried out on cellulose powder (Whatman) Fig. 1 indicates that rumen liquor was equally effective in producing almost complete solubilization of native cotton fibre.

Instability of the cellulolytic activity of rumen micro-organisms

Attempts to concentrate the cellulolytic enzyme of rumen liquor by centrifugation at 10,000*g* for 30 min. at 20° (initial temperature) led to varying losses

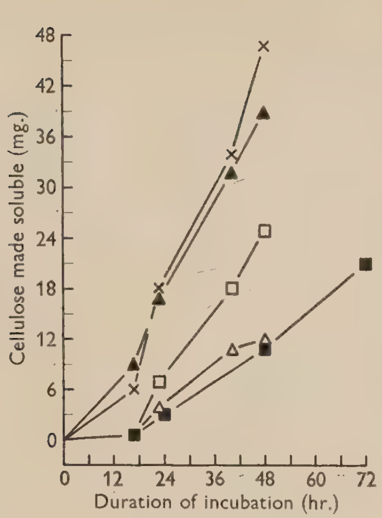

Fig. 1

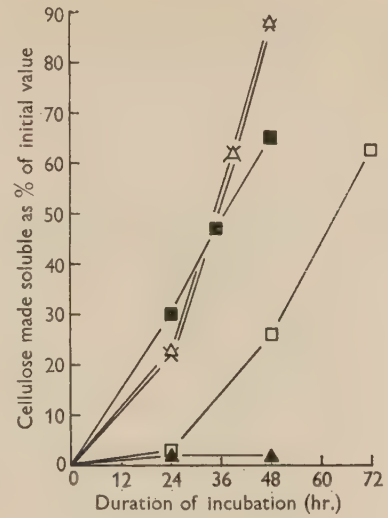

Fig. 2

Fig. 1. The effect of cellulose concentration on cellulolysis by rumen liquor. Cellulose powder (Whatman), initial weights 14 mg. △, 31 mg. □, 54 mg. ▲, 71 mg. ×. Native cotton fibres, initial weight 30 mg. ■. 1 ml. rumen liquor. Total volume to 8 ml. with bicarbonate salts buffer, pH 6·8.

Fig. 2. The effect of total assay volume and of shaking on cellulolysis by rumen liquor. Total volume of 4 ml. ■, of 8 ml. △, of 12 ml. ×. Effect of shaking in a total volume of 4 ml. 4 ml. shaken throughout ▲, 4 ml. shaken for first 24 hr. only □. Rumen liquor, 1 ml. Initial cellulose powder (Whatman), 50 mg.

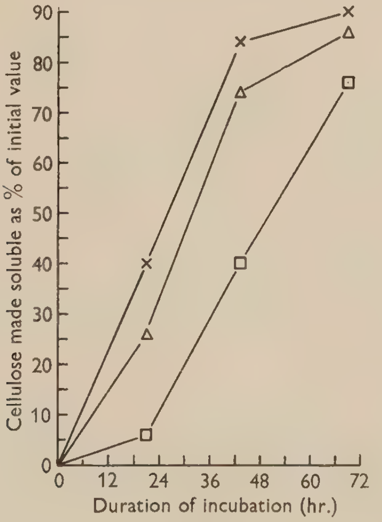

Fig. 3. The effect of rumen liquor concentration on cellulolysis. Rumen liquor, 0·4 ml. □, 0·8 ml., △, 2·0 ml., × ; initial cellulose powder (Whatman) 50 mg.; total volume 8 ml., bicarbonate-salts buffer, pH 6·8.

Table 1. *Effect of composition of the bicarbonate salts medium on cellulolysis by rumen liquor and by rumen liquor concentrates*

Standard cellulose assay unless stated otherwise.

Conditions with rumen liquor	Cellulose made soluble in 40 hr. as % of initial
1. Control, in bicarbonate + CO_2 buffer (pH 6·8) with KCl, $CaCl_2$, KH_2PO_4, $MgSO_4$, $(NH_4)_2HPO_4$	66
2. As (1) but not KCl.	66
3. As (1) but KCl at 25 × concentration	52
4. As (1) but no $(NH_4)_2HPO_4$	18
5. As (1) but no $MgSO_4$	20
6. As (1) but containing only bicarbonate, $MgSO_4$ and $(NH_4)_2HPO_4$	60
7. As (6) but containing only bicarbonate and $MgSO_4$ (no $(NH_4)_2HPO_4$)	16
8. As (6) but containing only bicarbonate and $(NH_4)_2HPO_4$ (no $MgSO_4$)	13
9. As (6) but containing $MgSO_4$ at 0·2 × concentration	36
10. As (6) but containing $MgSO_4$ at 5 × concentration	48
11. As (6) but containing $MgSO_4$ at 25 × concentration	46
12. As (1) but containing $CaCl_2$ at 30 × concentration	0
13. As (1) but containing no $CaCl_2$	60
14. As (1) but containing no KH_2PO_4	60
15. As (1) but containing 0·013 M-acetic acid (final concentration) pH 6·8	69
16. As (1) but no bicarbonate and no $(NH_4)_2HPO_4$ (NH_4Cl replaces $(NH_4)_2HPO_4$; KH_2PO_4 + NaOH + N_2 (pH 6·8) replaces bicarbonate-CO_2 (pH 6·8))	0
17. As (16) but containing also bicarbonate + CO_2 at same concentration as (1) i.e. total PO_4 concentration now as in (16) i.e.; 11 × control (1)	56
18. As (1) but containing 0·03 % Na_2S (final)	66
Conditions with concentrates (R.P.)	
1. Control, in bicarbonate + CO_2 buffer pH 6·8 with KCl, $CaCl_2$, KH_2PO_4, $MgSO_4$ and $(NH_4)_2HPO_4$	88
2. As (1) but in addition 0·03 % Na_2S (final)	86
3. As (1) but in addition 0·05 M-$K_3Fe(CN)_6$ (final)	1
4. As (1) but in addition 0·13 % gelatin (final)	88
5. As (1) but in addition 0·25 M-sucrose (final) and 0·02 M-NaCl (final)	40
6. As (1) but in addition 0·02 M-NaCl (final)	88
7. As (1) but in addition 0·1 M-NaCl (final)	46
8. As (1) but at pH 6·6	86
9. As (1) but at pH 6·6 and in addition, rumen liquor supernatant fluid (R.L.S.)	86
10. As (1) but at pH 5·9	0

in activity. Thus, after centrifugation, the precipitated micro-organisms and the supernatant fluid of rumen liquor were: (1) remixed in the same vessel, or (2) the supernatant fluid was rejected and replaced by the standard bicarbonate + salts buffer (pH 6·8). The two suspensions were immediately assayed on cellulose powder (Whatman); the first treatment produced 66 % hydrolysis of the cellulose and the second gave only 25 % hydrolysis. A control which had remained untreated at 20° for 30 min. gave 87 % hydrolysis of cellulose powder

(Whatman), whilst the clear supernatant fluid which had been removed had no action on the cellulose. When centrifugation was done at 0° and in presence of sodium sulphide (0·03 %) as in the standard procedure for making the deposit of rumen organisms (see Methods) followed by treatment (2) above, the suspension effected 54 % hydrolysis of cellulose.

An alternative procedure for fractionation and concentration involved the use of a glass tube 3·0 cm. diameter × 150 cm. which was completely filled with rumen liquor (pH 6·7) and closed. The liquor was then allowed to settle for 25 hr. at 1°, during which time three layers were produced. They were carefully separated by pipette and analysed for cellulolytic activity by the standard procedure. A control was subjected to the same treatment, but re-mixed without separation of phases and then assayed on cellulose powder (Whatman). No loss of cellulolytic activity resulted. The three layers which were separated provided the following data: (1) an upper layer with low activity and large volume (19 % of total cellulolytic activity of the control); (2) second layer of high activity and small volume (15 % of cellulolytic activity of control); (3) a precipitate of high activity and small volume (9 % of cellulolytic activity of control). Thus the total recovery of cellulolytic activity from the three fractions amounted only to 43 % of that of the original unfractionated rumen liquor and no single fraction offered any source of a concentrated form of enzyme. The losses were not due to removal of soluble essential factors from the precipitated micro-organisms as was shown by addition of the supernatant fluid (R.L.S.) to these deposited organisms, when no improvement followed.

Various antiseptics were added to the standard assay mixture containing rumen liquor. It was found that NaF (final concentration 0·02 M) acted immediately, whether added at 0 hr. or after 24 hr. fermentation of the rumen liquor + cellulose mixture. When added at the beginning of fermentation, cellulolysis in 48 hr. was decreased from 50 % (control in absence of NaF) to 2 % of the initial cellulose powder (Whatman). When NaF was added after 24 hr. of fermentation, the amount of cellulose powder hydrolysed (48 hr.) amounted to only 10 % as compared with the control values of 7 % at 24 hr. and of 50 % at 48 hr. Thus, in the hydrolysis of the cellulose powder by rumen liquor + NaF, no more than 2 % or 3 % of the available cellulose was solubilized in 48 hr. or 24 hr., respectively. It appears that the principal enzymic action in presence of NaF is on associated polysaccharide material rather than on true cellulose. Similar experiments were carried out using thymol at a concentration sufficient to saturate the medium in the standard assay in presence of rumen liquor. The incubation was prolonged to 72 hr. to obtain a greater possible hydrolysis. In absence of thymol 84 % of the cellulose powder (Whatman) disappeared, whereas thymol produced complete loss of the cellulolytic activity of rumen liquor. Under identical conditions toluene (12 %, v/v) permitted only 4 % hydrolysis of cellulose powder. Hence, all these inhibitors produced almost total loss of true cellulolytic power.

Role of protozoa in cellulolysis by rumen liquor

In view of the alleged role of protozoa in cellulose metabolism by rumen liquor a detailed examination was made to determine their possible importance. In collaboration with Drs A. E. Oxford and M. Eadie (for oligotrichs) and Dr G. Thomas (for holotrichs) of this Institute, representative organisms of the holotrich and oligotrich population normally frequenting the rumen were examined for their cellulolytic activity.

The holotrich protozoa were isolated by the method of Masson & Oxford (1951) and of Oxford (1951). The concentrated protozoa were then washed several times, by decantation, with the acetate buffer of Sugden (1953). Subsequent disintegration of the organisms by freezing and thawing followed by centrifugation and filtration through a bacteriological glass filter provided a pale yellow extract of pH value 6·0. This extract, however, exhibited no significant cellulolytic action on cellulose powder nor on hydrocellulose (from 11 N-HCl). The absence of cellulase activity from rumen holotrichs is not altogether unexpected, for such organisms are more adapted to the metabolism of starch and soluble carbohydrates. On the other hand, oligotrichs are believed to be concerned with the digestion or ingestion of cellulose.

In order to avoid the difficulties encountered in separating the holotrichs from the oligotrichs of rumen liquor, advantage was taken of the relative paucity of holotrichs in a particular animal (Sheep no. 91). Oligotrichs were isolated by the procedure of Sugden (1953) and washed repeatedly with acetate + sulphide buffer (pH 6·8), until a 60-fold concentration of the original rumen liquor organisms was achieved. The protozoal fraction was then assayed for cellulolytic activity on cellulose powder in: (1) the above acetate + sulphide buffer as normally employed for oligotrich metabolism; (2) the $NaHCO_3$ + sulphide buffer which, as previously shown, is well suited for cellulolysis by rumen liquor bacteria in the standard 'cellulase' assay. No extraneous source of N was supplied (e.g. R.L.S.). After incubation for 5 days at 37°, 10 % of the cellulose powder (Whatman) was solubilized in the acetate medium, as compared with 86 % in the $NaHCO_3$ medium.

More oligotrich protozoal suspensions were now prepared in a similar manner, but from the rumen liquor of a different sheep (no. 44) which contained a normal mixed fauna of oligotrich and holotrich protozoa. The 14-fold concentrate (15 mg. dry wt./ml. suspension) so obtained was then examined for cellulolytic action on cellulose powder (Whatman), but with the addition of rumen liquor supernatant fluid (which itself is inactive on this cellulose powder) in both the acetate + sulphide and $NaHCO_3$ + sulphide buffers. The unconcentrated rumen liquor of sheep no. 44 was also examined for cellulolytic activity in $NaHCO_3$ + sulphide medium. Table 2 summarizes the results.

The pH value of the bicarbonate + sulphide cultures rose to a higher value than normally found in the standard rumen liquor assay. This effect probably resulted from the extended time of the fermentation (5 days). The protozoa survived in acetate buffer during the whole of the fermentation period, whereas in the bicarbonate medium they rarely lived more than 1 or 2 days.

A turbidity developed in the acetate and bicarbonate tubes during the fermentation and microscopic examination confirmed its bacterial nature. The bacterial flora presumably account for the almost total utilization (83 %) of cellulose in bicarbonate medium in contrast to the acetate medium, where, although active oligotrich protozoa were present, cellulolysis amounted only to 36 %. Hence, despite extensive washing and concentration procedures on the protozoa, with simultaneous dilution and rejection of bacteria, it would appear that sufficient bacteria survived, possibly engulfed by protozoa, to effect marked cellulolysis in bicarbonate, even after death of the oligotrichs.

Table 2. *Cellulolysis by washed suspensions of protozoa and by rumen liquor in acetate and in bicarbonate media*

Conditions: standard cellulase assay, but in bicarbonate + sulphide (0·02 %, w/v) or in acetate + sulphide (0·02 %) buffer (Sugden, 1953) with addition of rumen liquor supernatant fluid (R.L.S.) where indicated. Activity of protozoa refers to the presence of living (motile) organisms (+) or dead (−) on microscopic examination. 14-fold concentrate of protozoa used. Rumen liquor (R.L.), 1 ml. neat, added. Cellulose utilization given at 5th day of incubation. R.L.S. obtained from supernatant of R.L. after centrifugation 20,000 *g*, 1°, 30 min.

			pH value		Activity of protozoa after (days)			Cellulose used as % of available
Organisms	Medium	Additions	Initial	Final (5 days)	1	4	5	
Protozoa	Acetate + Na_2S	R.L.S.	6·7	7·1	+	+	+	36
Protozoa	$NaHCO_3 + Na_2S$	R.L.S.	7·1	8·5	+	−	−	83
R.L.	$NaHCO_3 + Na_2S$	None	7·0	8·3	.	.	.	80

The role of rumen liquor supernatant fluid in increasing the cellulolysis in acetate buffer threefold (10 % in sheep 91 to 36 % in sheep 44) appears difficult to define clearly, for at least two reasons. First, the rumen liquor fluid has no action on the breakdown of cellulose by rumen bacteria in bicarbonate buffer, as already shown above. Secondly, the rumen liquor fluid has no beneficial action in prolonging life of oligotrich protozoa (Sugden, 1953). However, it is not unlikely that rumen liquor fluid may stimulate the development in acetate buffer of those bacteria, ingested by or otherwise closely associated with protozoa, which are capable of attacking cellulose.

In order to determine whether cellulolysis by protozoal preparations was in fact due to bacterial contaminants, subsequent experiments were undertaken under aseptic conditions. Protozoal fractions were obtained as in previous experiments, but from a mixture of equal volumes of rumen liquor of sheep no. 44 (mixed protozoa) and of sheep no. 91 (almost holotrich-free). The 30-fold concentrate was then allowed to metabolize cellulose powder (Whatman) in presence of rumen liquor fluid in acetate + sulphide or bicarbonate + sulphide media, under sterile conditions (Table 3A). After 3 days of fermentation the protozoa appeared far more active in the acetate-containing medium, whereas the bicarbonate-containing medium contained many dead or sluggish ciliates. Examination of cultures from the acetate- and from the bicarbonate-media indicated the presence of Gram-negative cocci as the main bacterial flora.

Table 3. *Cellulolysis by washed suspensions of protozoa in acetate and in bicarbonate media*

Conditions: as in Table 2. C.P.W. = cellulose powder, Whatman; H.C.H. = hydrocellulose, from HCl treatment; Cellulose utilization given at 3rd day of incubation. Protozoa from 30-fold concentrate of R.L. Experiments A under sterile conditions.

				pH value		Activity of protozoa after (days)			Cellulose used as % of available
	Medium	Substrate	Additions	Initial	(3 days)	1	2	3	
A.	Acetate + Na_2S	C.P.W.	R.L.S.	6·7	7·3	+	+	+	7
	$NaHCO_3$ + Na_2S	C.P.W.	R.L.S.	7·0	7·6	+	+	+	42
B.	Acetate + Na_2S	C.P.W.	None	.	.	.	.	.	0
	Acetate + Na_2S	H.C.H.	None	.	.	.	.	.	0
	Acetate + Na_2S	C.P.W.	R.L.S.	.	.	.	.	.	23
	Acetate + Na_2S	H.C.H.	R.L.S.	.	.	.	.	.	0
	$NaHCO_3$ + Na_2S	C.P.W.	R.L.S.	.	.	.	.	.	81
	$NaHCO_3$ + Na_2S	H.C.H.	R.L.S.	.	.	.	.	.	77

Other workers (see 'Methods and substrates') have used a hydrocellulose (from HCl) in studies of protozoal cellulolysis. In order to see whether such a substrate was more susceptible to hydrolysis than was cellulose powder (Whatman) we examined the action of our protozoal preparations on a similar hydrocellulose. Protozoa were concentrated (30-fold) from mixed rumen liquors of sheep nos. 91 and 44 and allowed to metabolize cellulose powder (Whatman) or the hydrocellulose, in presence or absence of rumen liquor fluid (Table 3B). The protozoa were alive for at least 24 hr. after inoculation and all living organisms of *Metadinium medium* examined as well as some of the dead specimens contained ingested particles of cellulose. As found previously rumen liquor fluid assists the organisms (bacteria and/or protozoa) to hydrolyse cellulose in the acetate medium. Cellulolysis in the bicarbonate medium (therefore principally bacterial in nature) proved to be far more effective.

DISCUSSION

In work involving cellulolytic enzymes, either from the rumen or from alternative sources, it is important to emphasize that results are determined not only by the substrate, but also by the method of analysis. Different forms of cellulose, from native cotton fibres through the degraded states of hydrocellulose, precipitated cellulose and cellodextrins to the cellulose derivative carboxymethylcellulose, contain chains of similar chemical structure, but of very different lengths. Furthermore, carboxymethylcellulose is not even a true chemical cellulose, but rather a soluble cellulose derivative. The action of pure isolates of rumen organisms, or of mixed rumen micro-organisms, on any one form of 'cellulose' is no proof that other substrates are likewise susceptible. However, as we have shown, mixed rumen liquor micro-organisms produce almost complete solubilization of all forms of cellulose, from the simplest types up to the highly complex cotton fibres. These results were achieved by determination of the residual cellulose after treatment with micro-organisms and thus avoided the errors inherent in determinations of reducing sugars (see below).

The earlier difficulties connected with the gravimetric estimation of cellulose, namely the presence of filter-impeding bacterial products, appear to have been overcome by our technique, which employed selected solvents. The latter, even when used in amounts up to 5 times the volumes quoted in the standard technique are not detrimental. The washing process is so effective that the apparent endogenous cellulose of rumen liquor is negligible or in the case of concentrated preparations of the organisms from the liquor does not exceed 4 % of the cellulose used in the standard assay. We have also used the method in the determination of cellulolysis by pure strains of micro-organisms, e.g. *Sporocytophaga myxococcoides* (QM B482) and *Cellvibrio vulgaris* (QM B4) and *Myrothecium verrucaria* (I.M.I. 45541), with equally satisfactory results.

On the other hand, determination of cellulolysis by sugar formation from insoluble cellulose preparations makes no allowance for associated glucosans or xylans of relatively low molecular weight which may be solubilized under the assay conditions. In presence of enzyme(s) not necessarily 'cellulolytic', such non-cellulosic non-reducing fractions may well be hydrolysed to sugars. This effect assumes major importance when the observed cellulolysis never exceeds more than a fraction of the available cellulose. This appears to be the case in the work of Meites, Burrell & Sutton (1951), of Kitts & Underkofler (1954) and of Hungate (1942, 1943, 1950*b*). Meites *et al.* (1951), with ground filter-paper and rumen micro-organisms in presence of toluene, obtained in 120 hr. 4 % of hydrolysis as measured by sugar production. Kitts & Underkofler (1954) found that mixed rumen micro-organisms attacked soluble carboxymethylcellulose much more effectively than they did filter-paper or Alphacel (a cellulose powder). Thus, in presence of thymol, NaF or toluene rumen micro-organisms achieved, as determined by sugar analyses, approximately 60 % hydrolysis of susceptible links in carboxymethylcellulose and 14 % hydrolysis of ground filter-paper (calculations by present author) in 48 hr. Hungate (1942) obtained qualitative evidence (Benedict test and glucosazone formation) of the action of a protozoal extract on hydrocellulose in presence of toluene.

The small degree of hydrolysis of insoluble cellulose powders by rumen micro-organisms in presence of antiseptics thus found by the above workers (by estimating reducing sugars) and also by ourselves (4 % cellulolysis) represents, as we have shown, only a small fraction of their true (up to 84 %) cellulolytic capacity. Such low rates of cellulose hydrolysis may well represent enzymic breakdown of non-reducing fractions (xylans, glucosans) associated with relatively undegraded cellulose. Support for this proposal is seen in the chromatographic identification of xylose from an Alphacel + rumen micro-organism incubation (Kitts & Underkofler, 1954). Further evidence is given by the work of Huffman, Rabers, Spriesterbach & Smith (1955) who isolated short-chain polysaccharides from filter-paper, wood cellulose and cotton linters by aqueous extraction.

The protozoal extract of Hungate described above may also have acted on a minor constituent of the cellulose or alternatively the activity of the extract might have been bacterial in origin. We have shown that, despite extensive washing of protozoa, sufficient bacteria to produce marked cellulolysis may remain. This might involve the cellulose seen to be ingested in both living and dead oligotrich protozoa and perhaps true 'cellulase', secreted by ingested bacteria, is more stable within the protozoa than in the medium. The slight degree of hydrolysis of cellulose obtained in the work cited was presumably effected by an enzyme different from that which hydrolyses true cellulose (cf. Reese, Siu & Levinson, 1950). The former enzyme system, in rumen micro-organisms, appears to be relatively stable to agitation (cf. Meites *et al.* 1951) and to antiseptics. However, the true cellulolytic activity of rumen micro-organisms is, as we have shown, extremely sensitive to a variety of conditions, including agitation, antiseptics and all attempts at concentration. From the present results on cellulolysis it seems evident that hydrolysis is much greater in a bicarbonate medium (conducive to bacterial growth) than in acetate buffer (as used for protozoal cultivation). Whether or not the protozoa survived in either medium appears unimportant for cellulolysis provided the associated bacteria flourish. This suggests that rumen protozoa owe much, if not all, of their cellulolytic activity to the presence of their associated bacterial commensals.

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REFERENCES

AITKEN, R. A., EDDY, B. P., INGRAM, M. & WEURMAN, C. (1956). The action of culture filtrates of the fungus *Myrothecium verrucaria* on β-glucosans. *Biochem. J.* 64, 63.

BASU, S. N. & PAL, P. N. (1956). Unfavourable effect of shaking on fungal cellulases. *Nature, Lond.* 178, 312.

BRYANT, M. P. & BURKEY, L. A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* 36, 205.

ELSDEN, S. R. (1945). The fermentation of carbohydrates in the rumen of the sheep. *J. exp. Biol.* 22, 51.

HIGA, H. H., O'NEILL, R. D. & JENNISON, M. W. (1956). Partial purification of cellulase from a wood rotting Basidiomycete. *J. Bact.* 71, 382.

HUFFMAN, G. W., RABERS, P. A., SPRIESTERBACH, D. R. & SMITH, F. (1955). Nature of a hemicellulose extracted from cellulose with water. *Nature, Lond.* 175, 990.

HUNGATE, R. E. (1942), The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. *Biol. Bull., Woods Hole*, 83, 303.

HUNGATE, R. E. (1943). Further experiments on cellulose digestion by protozoa in the rumen of cattle. *Biol. Bull., Woods Hole*, 84, 157.

HUNGATE, R. E. (1950*a*). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* 14, 1.

HUNGATE, R. E. (1950*b*). Mutualisms in protozoa. *Annu. Rev. Microbiol.* **4**, 53.

JERMYN, M. A. (1952). Fungal cellulases. 1. General properties of unpurified enzyme preparations from *Aspergillus oryzae*. *Aust. J. sci. Res.* B, **5**, 409.

KITTS, W. D. & UNDERKOFLER, L. A. (1954). Digestion by rumen micro-organisms. Hydrolytic products of cellulose and the cellulolytic enzymes. *J. Agric. Food Chem.* **2**, 639.

MASSON, F. M. & OXFORD, A. E. (1951). The action of the ciliates of the sheep's rumen upon various water soluble carbohydrates, including polysaccharides. *J. gen. Microbiol.* **5**, 664.

MEITES, S., BURRELL, R. C. & SUTTON, T. S. (1951). Factors influencing the *in vitro* digestion of cellulose by rumen liquor in presence of an antiseptic. *J. Anim. Sci.* **10**, 203.

NISHIZAWA, K. & KOBAYASHI, T. (1953). Cellulose splitting enzymes. 11. Mode of action of *Irpex* cellulase on its substrates. *J. Agric. chem. Soc. Japan.* **27**, 239.

OXFORD, A. E. (1951). The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. *J. gen. Microbiol.* **5**, 83.

PHILLIPSON, A. T. (1953). Digestion of cellulose by the ruminant. In *Biological Transformations of Starch and Cellulose. Symp. biochem. Soc.* **11**, 63.

PHILLIPSON, A. T. & CUTHBERTSON, D. P. (1956). Modern concepts of rumen digestion and metabolism. *7th Int. Congr. Anim. Husb., Madrid, Subject* **6**, p. 1.

REESE, E. T., SIU, R. G. H. & LEVINSON, H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J. Bact.* **59**, 485.

SIJPESTEIJN, A. K. (1951). On *Ruminococcus flavefaciens*, a cellulose decomposing bacteria from the rumen of sheep and cattle. *J. gen. Microbiol.* **5**, 869.

SIU, R. G. H. (1951). *Microbial Decomposition of Cellulose.* New York: Reinhold Publ. Corporation.

SUGDEN, B. (1953). The cultivation and metabolism of oligotrich protozoa from the sheep's rumen. *J. gen. Microbiol.* **9**, 44.

THOMAS, R. (1956). Fungal cellulases. VII. *Stachybotrys atra*: production and properties of the cellulolytic enzyme. *Aust. J. biol. Sci.* **9**, 159.

WALSETH, C. S. (1952). Occurrence of cellulases in enzyme preparations from micro-organisms. *Tech. Pap., Pulp. Pap. Ind. N.Y.* **35**, 228.

WHITAKER, D. R. (1953). Purification of *Myothecium verrucaria* cellulase. *Arch. Biochem. Biophys.* **43**, 253.

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Halliwell, G. (1957). *J. gen. Microbiol.* 17, 166–183

Cellulolytic preparations from Micro-organisms of the Rumen and from *Myrothecium verrucaria*

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SUMMARY: A study has been made of the degradation of different forms of cellulose ('cellulolysis'), comprising soluble substituted derivatives (carboxymethylcellulose), insoluble cellulose powder, swollen cellulose, hydrocellulose and native cotton fibres, by concentrated suspensions of mixed rumen micro-organisms and by the aerobic fungus *Myrothecium verrucaria.* Mixed rumen micro-organisms are shown to be one of the most powerful sources of cellulolytic enzyme, in that they produce almost complete solubilization of all the above forms of cellulose in a relatively short period (3 days). Enrichment cultures of rumen micro-organisms were prepared by growing concentrates of mixed micro-organisms on cellulose powder. Cellulolysis was followed by determining the cellulose disappearance, formation of cellulolytic activity and gas evolution. Freeze-dried powders and their derived acetone powders obtained from washed concentrated supensions (non-enrichment cultures) of mixed rumen micro-organisms solubilized up to 80 % of insoluble cellulose powder. Cell-free extracts were isolated: (*a*) from concentrated suspensions and from freeze-dried powders of rumen micro-organisms by extraction with butanol; (*b*) from concentrated suspensions of enrichment cultures by grinding with alumina at low temperature. The butanol extracts solubilized cellulose powder to a small extent (10 % solubilization) and were more effective against the soluble carboxymethylcellulose. In contrast, the alumina extracts were more active against insoluble cellulose (30 % solubilization) but were only weakly active against carboxymethylcellulose. Cell-free filtrates from *Myrothecium verrucaria*, an aerobic fungus much used in work on cellulose metabolism, were shown to possess cellulolytic properties very similar to those of alumina extracts from enrichment cultures of rumen micro-organisms. The significance of the results is discussed in relation to the mode of breakdown of cellulose.

Despite much work, little appears to be known about the conditions governing the biological breakdown of cellulose. Many workers have obtained extracts, from animal, vegetable and microbial sources, with only feeble activity on insoluble forms of cellulose, and have turned to using as substrates extensively degraded cellulose or even the soluble substituted cellulose derivatives. Whilst such substrates usually prove to be more susceptible to hydrolysis, they leave the primary question unanswered, namely, how is true or insoluble cellulose hydrolysed for utilization by a select group of micro-organisms? Numerous examples of 'cellulase' preparations which fail to attack insoluble cellulose with any significance are given in an excellent monograph by Siu (1951). More recent cases are quoted by Halliwell (1957) where calculations show that hydrolysis of cellulose powder or cotton never exceeds more than about 6 % of the substrate.

Despite the feeble action demonstrated by these extracts, the parent organism, whether fungus or bacterium, is often capable of producing complete solubilization of most forms of cellulose. This difference has been attributed to the presence of at least two enzymes, the first of which attacks more complex and insoluble molecules and thus renders them sensitive to degradation by a further enzyme (Reese, Siu & Levinson, 1950). Hence, truly cellulolytic micro-organisms containing the two (or more) enzymes are capable of hydrolysing native cellulose as well as degraded celluloses and the soluble cellulose derivatives, e.g. carboxymethylcellulose. Non-cellulolytic organisms are said to lack this primary enzyme and hence fail to attack insoluble cellulose, although soluble cellulose derivatives are often readily hydrolysed. On the other hand, Whitaker (1953) believed that a single enzyme is responsible for hydrolysis of native cellulose and carboxymethylcellulose. However, his results with a purified concentrate of cellulase from *Myrothecium verrucaria* indicate not more than 2 % hydrolysis of cotton linters no matter how much enzyme was added.

In rumen cellulolysis the problem appears to be further complicated by the necessity for anaerobiosis. No one has yet obtained extracts or even pure strains of micro-organisms which exhibit significant cellulolysis of undegraded cellulose. Invariably, workers have employed hydrolysed and degraded forms of cellulose.

In work on cellulose degradation in the rumen it is preferable to employ mixed micro-organisms rather than isolated pure strains because (1) there is less divergence from *in vivo* cellulolytic activity, and (2) our mixed cultures, unlike pure strains of other authors, act relatively rapidly and completely on all forms of insoluble and undegraded cellulose tested by us (possibly because more than one strain of micro-organism is essential for complete cellulolysis).

In the work presented here an attempt has been made to determine the conditions controlling the hydrolysis of cellulose and the production of 'cellulase'. To this end we have used two sources of enzyme: (1) the anaerobic system of mixed rumen micro-organisms; (2) the aerobic fungus, *Myrothecium verrucaria*. Cellulolysis has been determined gravimetrically from the loss in weight of the residual cellulose rather than by determination of the increase in reducing sugar value. The latter procedure, as noted in the Discussion, can lead to false and high values for hydrolysis.

METHODS

Substrates. These were identical to those reported earlier (Halliwell, 1957). Sodium carboxymethylcellulose, a soluble cellulose derivative with a degree of substitution of about 0·5, was generously supplied as Cellofas B by Imperial Chemical Industries Ltd.

Source of cellulase. Rumen liquor (R.L.), rumen liquor supernatant fluid (R.L.S.) obtained by high speed centrifugation and rumen liquor concentrates (R.P.) were prepared as described by Halliwell (1957).

Preparation of enrichment cultures of rumen micro-organisms. A bicarbonate + salts medium (see Halliwell, 1957), with cellulose powder (Whatman), sodium sulphide for anaerobiosis, R.L.S. and yeast extract to supply additional nutrients, and an inoculum of an eight-fold concentrate (R.P.) of rumen micro-organisms, were incubated at 37° under CO_2 for the times stated in the text. The fermentation vessel consisted of a conical flask fitted with a Bunsen valve. For gas analysis experiments, the exit tube of the valve was replaced by a direct glass connexion to an inverted measuring cylinder over dilute hydrochloric acid. The composition of the medium is tabulated below:

Composition of enrichment medium (%, w/v); $NaHCO_3$, 1·47; KCl, 0·04; KH_2PO_4, 0·018; $MgSO_4, 7H_2O$, 0·03; $(NH_4)_2HPO_4$, 0·09; $CaCl_2, 6H_2O$, 0·06; Cellulose powder (Whatman) 0·4, 1·2, 2 or 3·0; Yeast extract, Difco Bacto, 0·03; $Na_2S, 9H_2O$, 0·02; R.L.S., 20 (v/v); Inoculum, eight-fold concentrate of R.L. micro-organisms, 5 (v/v); pH value with CO_2, adjusted to 6·8.

Preparation of enzyme extracts from enrichment cultures of rumen micro-organisms using alumina. Extracts capable of producing appreciable hydrolysis of insoluble cellulose were obtained by centrifuging enrichment cultures, after a suitable period of fermentation, at 20,000 *g* for 20 min. at 1° and washing the precipitated organisms with 0·02 % $Na_2S, 9H_2O$ (pH 7·2). The rumen liquor concentrate thus obtained was cooled to 0° for 1 hr. and then ground in portions at that temperature in a previously cooled mortar with a total of 3 times its weight of dried washed alumina for 15 min. (McIlwain, 1948). The ground powder was then extracted with 3 vol. of sulphide + phosphate buffer (0·02 %, w/v, $Na_2S, 9H_2O$ in 0·067 M-Na_2HPO_4 + 0·067 M-KH_2PO_4, pH 7·2) centrifuged at 25,000 *g* for 30 min. at 1°, the supernatant fluid removed and centrifuged for 20 min. The final optically clear yellow supernatant fluid (pH 7·2) possessed appreciable activity with insoluble cellulose powder (Whatman, C.P.W.), up to 30 % being solubilized, but the preparation had only feeble activity (compared with the butanol preparation described below) on carboxymethylcellulose.

Preparation of enzyme extracts from rumen micro-organisms with butanol. Morton's technique (1950) was adapted to prepare enzyme extracts from rumen liquor concentrates (R.P.). The latter, obtained by centrifugation as in the previous paper, were cooled to 1° and stirred at that temperature with about 0·4 vol. of *n*-butanol (A.R.) for 15 min., after which the whole suspension was centrifuged at 20,000 *g* for 20 min. at 1°. The aqueous phase containing butanol was carefully separated from over- and under-lying layers and re-centrifuged to provide a clear orange-coloured supernatant fluid (pH 7·6). This enzyme preparation exhibited appreciable activity on carboxymethylcellulose (C.M.C), but did not cause more than 10 % hydrolysis of cellulose powder (Whatman) and did not attack cotton fibres at all.

Preparation of freeze-dried powders from rumen micro-organisms and of derived acetone powders. These powders retained much of the true cellulolytic capacity of the parent micro-organisms, and were more effective than either butanol extracts or alumina extracts, in that they produced almost complete solubilization (up to 80 %) of cellulose powder (Whatman). The preparation of this freeze-dried powder is outlined below, where 'recovery of activity' refers to the total 'cellulase' value (proportional to the weight of cellulose solubilized by unit original volume) as determined by the standard assay on cellulose powder in bicarbonate buffer (see below):

Centrifuge Rumen liquor at 1800 *g*, 1°, 30 min.

Precipitate 1 (17 % recovery of activity): suspend in 0·02 % $Na_2S, 9H_2O$ (pH 7·1) stir gently to wash, centrifuge as above for 55 min.

Supernatant fluid of large volume but of low activity; rejected

Precipitate 2 (60 % recovery of activity of precipitate 1): suspend in 0·02 % $Na_2S, 9H_2O$ (pH 7·1) adjust to pH 6·9 and freeze dry

Freeze-dried powder (100 % recovery of activity of precipitate 2 or 11 % recovery of activity of rumen liquor); Suspend in redistilled acetone at 1° (30 vol.), stir at 1° for 20 min.; centrifuge at 1800 *g*. for 15 min. at 1°

Precipitate of acetone powder: suspend in 0·02 % $Na_2S, 9H_2O$ (pH 7·1) to assay cellulase (50 % recovery of activity of freeze dried powder)

Supernatant fluid rejected

Myrothecium verrucaria cultures (I.M.I. 45541, Commonwealth Mycological Institute, Kew, Surrey) were grown on the salt medium of Saunders, Siu & Genest (1948) with cellulose powder (Whatman) as substrate. The cell-free culture fluid itself, unlike that of rumen liquor, possessed true cellulolytic activity against cellulose powder, without recourse to extraction of the organism. The fluid was first filtered through a grade 3 sintered glass filter to remove undissolved cellulose and mycelium, then centrifuged at 20,000 *g* for 30 min. at 1°, and subsequently sterilized by filtration through a sintered glass (5/3) bacteriological filter.

Determination of the cellulase activity of micro-organisms and of enzyme extracts. (a) Rumen micro-organisms and extracts were examined for 'cellulase' by the assay method described earlier (Halliwell, 1957), except that lyophilized powders, acetone powders and enzyme extracts were incubated for 72 hr. instead of 40 hr. as with organisms. (*b*) *Myrothecium verrucaria* cultures and enzyme filtrates were assayed in Sörensen phosphate buffer (pH 6·0; $0{\cdot}067\,\text{M-}Na_2HPO_4 + 0{\cdot}067\,\text{M-}KH_2PO_4$) with 30 or 50 mg. cellulose at 30° or at 50° in test tubes, 15 mm. × 150 mm., or in 25 ml. conical flasks. The total volume was made up to 6 ml. including 1 ml. or 2 ml. of microbial suspension or filtrate.

'*Cellulase*' *unit.* For comparative purposes in the text a 'cellulase unit' may be defined as the minimum amount which in an extract or suspension produces *c.* 70 % cellulolysis under standard conditions. One ml. of rumen liquor from hay-fed sheep, which hydrolyses about 70 % of the standard amount of cellulose powder in 40 hr. (Halliwell, 1957; Table 1), is the minimum volume with this activity. Concentrate-fed sheep may show much less activity in this period.

Method of determining degree of cellulose hydrolysis. This was done by gravimetric estimation of the residual cellulose (Halliwell, 1957). Control cellulose determinations containing insoluble cellulose and all reagents without micro-organisms or extracts therefrom, showed no loss of cellulose in periods up to 7 days at 37°. Assay tubes containing extracts of organisms and incubated overnight or longer were examined for the growth contamination of bacteria as films treated by Gram's method and by inoculation of samples into fresh cellulose + buffer mixture. The results were always negative. Further evidence of freedom from bacteria was shown by the constancy of pH value of the buffers used. Rumen micro-organisms produce a rapid change in pH value of the buffer in less than 15 hr. (see Results).

Cellulose weights. In the text initial cellulose weights are referred to as '% concentration'; this indicates g. insoluble cellulose/100 ml.

Reducing sugar determinations. Hydrolysis of carboxymethylcellulose (C.M.C.) to sugars was followed by a modification of the colorimetric procedure of Folin & Malmros (1929) adapted to the Spekker photoelectric absorptiometer (Hilger).

RESULTS

Properties of concentrates of rumen micro-organisms

In order to obtain concentrated preparations for enzymic investigations, rumen micro-organisms were centrifuged at 20,000 *g* for 20 min. (preferably at 1°) the supernatant fluid rejected and the precipitated organisms (R.P.) preserved at 1° until used. No loss of 'cellulase' occurred in R.P. stored at this temperature up to 3 days. Some of the general properties of R.P., particularly in relation to the instability of its true cellulase, have been described earlier (Halliwell, 1957, Table 1). Several washing methods were examined by the cellulase assay procedure in order to determine the most suitable way to obtain relatively clean preparations which were active against cellulose. As Table 1 shows, the most suitable process for obtaining concentrated washed suspensions of organisms involved the use of 0·02 % (w/v) sodium sulphide in 0·16 M-sodium chloride (pH 6·9). The consistently low value obtained by washing with cysteine + phosphate buffer is possibly an effect associated with potassium phosphate in absence of ammonium phosphate (which supports growth) at this pH value. A similar effect was observed with phosphate as a washing medium before freeze-drying of concentrated suspensions of rumen micro-organisms. The effect was not examined further; it may be noted that Sijpesteijn & Elsden (1952) found that dipotassium hydrogen phosphate exerted an inhibitory action on the muscle succinic dehydrogenase system.

The 'cellulase' of rumen micro-organisms appears to be extremely unstable, as observed in attempts at concentration which involved exposure of large surfaces to the air or to elevated temperatures. Thus, representative samples of rumen liquor were treated as follows: (1) separate samples were centrifuged successively in a single centrifuge tube at 15000*g* for 30 min. (uncooled; initial temperature of 18°); the supernatant fluid was rejected and the precipitated micro-organisms covered with fresh rumen liquor and re-centrifuged; (2) the samples were treated in separate centrifuge tubes by centrifuging as above, after which the supernatant fluid was rejected and the precipitate stored at 1°; (3) samples were treated exactly as in (2), but at 1° throughout and with 0·02 % sulphide added to the rumen liquor. Each treatment was repeated and the precipitated organisms pooled until a three-fold concentration of the original rumen micro-organisms had been achieved. The pooled concentrates from each treatment, when examined by the standard cellulase assay, gave 4, 60 and 90 % hydrolysis of cellulose respectively, and thus indicated the advantages to be gained by maintaining a low temperature and reducing conditions.

Table 1. *The effect of washing procedures on recovery of cellulase activity from micro-organisms deposited (R.P.) by centrifugation of rumen liquor*

R.L.S. = supernatant fluid after centrifugation of rumen liquor

Treatment	$(NH_4)_2HPO_4$ absent	$(NH_4)_2HPO_4$ present
	Cellulolysis* (by standard assay method) (%)	
(1) Organisms resuspended in R.L.S.	Not done	85
(2) Organisms resuspended in $NaHCO_3$ + salts buffer containing 0·02 % Na_2S (pH 6·8)	Not done	70
(3) Organisms resuspended in $NaHCO_3$ + salts buffer containing 0·02 % Na_2S (pH 6·8) after:		
(*a*) Washing with Sörensen phosphate buffer (pH 6·9) and recentrifuging	44	44
(*b*) After washing with 0·004 M-cysteine in Sörensen phosphate buffer (pH 6·4)	19	71
(*c*) After washing with 0·004 M-cysteine + 0·16 M-NaCl (pH 6·4)	43	61
(*d*) After washing with 0·02 % Na_2S + 0·16 M-NaCl (pH 6·9, 6·4)	66	81

* Cellulolysis corrected for endogenous blank of deposited organisms.

Action of concentrated preparations of micro-organisms on various forms of cellulose

Fig. 1 illustrates the capacity of concentrated preparations of micro-organisms from rumen liquor (R.P.) to hydrolyse most forms of cellulose, from the degraded forms such as hydrocellulose to chemically untreated cotton fibres. Hydrocellulose and cellulose powder (Whatman) were hydrolysed to about the same degree (90 %), with a higher initial rate of attack on cellulose powder. Swollen cellulose powder was attacked even more rapidly, but ultimately left a resistant residue amounting to *c.* 20 % of the initial cellulose. In contrast, cotton fibres underwent only about 70 % solubilization in the same period (70 hr.). Dewaxing of the fibres by ethanol-ether-NaOH extraction gave no significant increase in the rate of cellulolysis. In all cases the initial rapid cellulolysis was followed by a much slower phase approaching complete degradation of substrate. The pH changes during the fermentation are shown in Fig. 2. Hydrolysis of the soluble cellulose derivative carboxymethylcellulose to sugars by R.P. was difficult to follow except in presence of bacterial growth inhibitors to prevent conversion to fatty acids. However, as shown below, cell-free extracts of R.P., obtained by butanol treatment, readily attacked carboxymethylcellulose (Fig. 3).

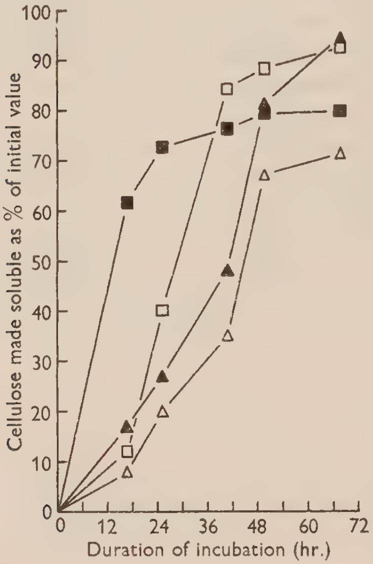

Fig. 1

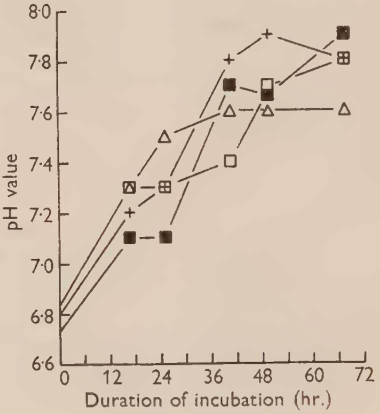

Fig. 2

Fig. 1. Action of rumen liquor concentrates (R.P.) on different forms of cellulose. Standard cellulase assay. Cellulose solubilized as % of initial weight; cotton fibres △, cellulose powder (Whatman) □, swollen cellulose powder (Whatman) ■, hydrocellulose ▲.

Fig. 2. Changes in pH value during fermentation of different forms of cellulose by rumen liquor concentrates (R.P.). Conditions as in Fig. 1. Cotton fibres ■, cellulose powder (Whatman) △, swollen cellulose powder (Whatman) +, hydrocellulose □.

Cellulolytic extracts from concentrated suspensions of rumen micro-organisms (*non-enriched*)

Several attempts were made to obtain active cellulolytic extracts from concentrated suspensions (R.P.), after carefully adjusting conditions to pH 7·0 and 1° in presence of 0·02 % (w/v) sulphide. Grinding (for 15 min.) of such R.P. preparations in presence of R.L.S. in a glass homogenizer diminished the total cellulase value to 70 % of the value of an unhomogenized preparation, whereas repeated freezing and thawing of R.P. from −30° to +30°, over a period of 2 days, produced no change in the total cellulolytic activity. However, both these treatments did not release enzyme to centrifuged supernatant fluid fractions. R.P. suspensions were also treated in the Mickle disintegrator, (Mickle, 1948) with ballotini beads (grade 12; Chance Bros. Ltd.,) at pH 6·2 for 30 min. at 1°, and provided material with up to four-fold the activity of the initial R.P. suspension, whereas similar treatments at pH 8·2 retained only 30 % of the total original cellulase activity. Acetone powders from R.P., prepared in the usual manner, resulted in almost complete loss of cellulolytic activity when assayed on cellulose powder (Whatman) or on hydrocellulose. The supernatants centrifuged at 20,000***g*** from the Mickle disintegrates never gave more than 5 % hydrolysis of cellulose powder (Whatman), whilst an aqueous extract from the acetone powder was completely inactive. Grinding of R.P. with alumina at 1° and extraction with Sörensen phosphate buffer containing 0·02 % (w/v) sodium sulphide over the pH range 6·7–7·5 provided optically clear yellow extracts which were devoid of cellulase activity. Butanol preparations of R.P. (see Methods) were more successful, but such extracts never gave more than about 10 % hydrolysis of cellulose powder (Whatman), although they acted quite rapidly on the soluble cellulose derivative carboxymethylcellulose (25 % hydrolysis of susceptible links in 18 hr.; Fig. 3).

Preparation and properties of freeze-dried powders from rumen micro-organisms and of derived acetone powders

By careful control of conditions it was possible to obtain a 'standard' freeze-dried powder, and from this an acetone powder, in both of which the 'cellulase' was sufficiently potent to solubilize up to 83 % of the initial amount of cellulose powder (Whatman) (see 'Methods' for preparation). To obtain active preparations it was necessary to complete the separations at 1° and to avoid undue exposure to air (0·02 %, w/v, $Na_2S, 9H_2O$ was used in washing procedures). On the large scale (4 l.) described under Methods, much cellulase activity was lost in the primary low speed (1800***g***) centrifugation. Some of this activity was recovered by centrifugation of the rejected supernatant fraction at high speed, but the minor improvement in specific activity (cellulase activity/g. dry weight freeze dried powders) of the ultimate freeze-dried product did not warrant the effort involved. The presence of sulphide was most critical at the actual freeze-drying stage because its absence caused marked losses on drying. Direct lyophilization of rumen liquor, without the preliminary concentration and washing, yielded dried powders with an over-all recovery of cellulase similar to that of the standard powder; however, the specific activity was only about one-twelfth of that of the standard dried product. The pH value at which lyophilization is done must likewise be carefully chosen and as Fig. 4 indicates the optimal value required in order to obtain high 'cellulase: dry-weight ratios' is about pH 7·4. Preparations were adjusted with HCl or NaOH to different pH values and the effect of the final pH value of the preparation after drying was determined (Fig. 4).

Another kind of freeze-dried powder was prepared by centrifuging rumen liquor for a short period (300***g*** for 30 sec. at 1°) to remove larger particles. The cloudy supernatant fluid was then fractionated by centrifugation and washing exactly as in the standard procedure described above. Freeze-dried powders obtained by this modified procedure looked more homogeneous and gave more uniform supensions, but never possessed more than half of the total cellulase activity of the standard dried powder. The latter appeared to be reasonably stable in that it still retained up to one-third of its total initial cellulase activity after 7 weeks at 1° under vacuum over calcium chloride.

It was possible that the freeze-dried powders contained viable micro-organisms and in order to eliminate this possibility acetone powders were prepared from lyophilized preparations by the usual techniques (see 'Methods' under 'Preparation of freeze-dried powders'). Approximately half of the total cellulase content of the parent freeze-dried product was recovered in the acetone powder which itself was capable of hydrolysing up to 80 % of cellulose powder (Whatman) in the standard cellulase assay. The freeze-dried powders appeared to be much more stable in aqueous suspension than did acetone powders prepared from freeze-dried powders. Thus, suspensions of a freeze-dried powder (final natural pH *c.* 6·7) in: (1) de-aerated water; (2) 0·01 % (w/v) $Na_2S,9H_2O$, pH 7·0 (adjusted with HCl); (3) 0·01 % (w/v) sulphide, pH 10·4, followed by a standard cellulase assay after each treatment, gave 80, 80, and 72 % hydrolysis of cellulose powder (Whatman), respectively. Even after incubation at pH 9·7 for 3 hr. at 30° in an 0·01 % (w/v) sulphide suspension, freeze-dried powders were still capable of producing 74 % hydrolysis of cellulose powder (Whatman). In contrast, acetone powders (prepared from freeze-dried powders) appeared to be far more sensitive to alkali. Thus a suspension of an acetone powder in 0·01 % (w/v) sulphide at pH 10·4, gave only half the cellulolytic activity of the same powder suspended in sulphide at pH 7·0, although, due to buffering action of the acetone powder, the final pH value was 6·7 in both cases.

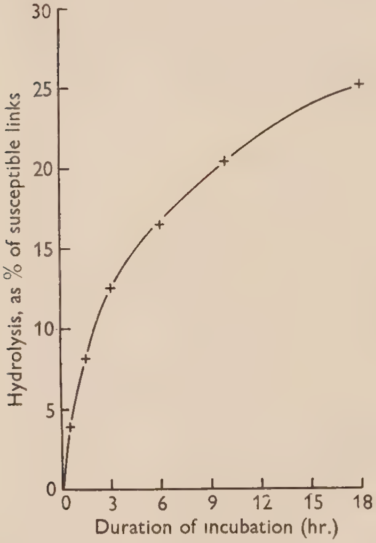

Fig. 3

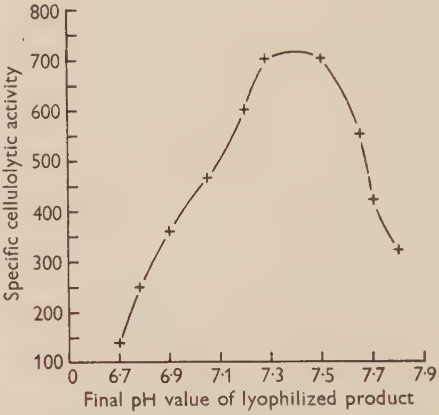

Fig. 4

Fig. 3. Hydrolysis of carboxymethylcellulose by cell-free butanol extract of rumen liquor concentrates (R.P.). Conditions, 0·2 ml. butanol extract on carboxymethylcellulose (10 mg.) in Sörensen phosphate buffer (pH 5·5). Total assay volume 4 ml.; 37°. Susceptible links calculated from Spurlin (1939).

Fig. 4. Effect of pH value at freeze-drying on recovery of cellulase activity. Activity given as specific activity/g. freeze-dried powder.

Stimulatory action of rumen liquor supernatant fluid on cellulolysis by lyophilized powders

The cellulase assays of the dried powders described above, were completed in presence of the high speed supernatant fluid (R.L.S.) from rumen liquor. R.L.S., which itself was devoid of cellulase, when added to the assay system increased the activity of freeze-dried preparations eight-fold in the standard cellulase assay. When R.L.S. was heated (by immersion of its container in boiling water for 1 min.) before addition to the assay system, the activity of lyophilized powders was raised only two-fold.

Certain fatty acids were found to stimulate the growth of a rumen isolate on ball-milled filter paper and on glucose (Bryant & Doetsch, 1955), and also to stimulate cellulolysis by mixed rumen micro-organisms (Bentley, Lehmkuhl, Johnson, Herschberger & Moxon, 1954). In order to determine whether such acids accounted for the activating effect of R.L.S. on our freeze-dried powders, *n*-valeric and *iso*valeric acids were used, singly and together, at 10, 100 and 500 % of the concentrations suggested by Bentley *et al.* (1954). These acids had no stimulating effect on the cellulase activity of our freeze-dried preparations. This result was not unexpected if the principal action of the fatty acids were supposed to be on growth of micro-organisms, rather than on cellulolysis.

Preparation of cellulolytic extracts from lyophilized powders of rumen micro-organisms and derived acetone powders

Autolysis of a freeze-dried powder suspension in 0·01 % (w/v) sulphide at pH 7·0 or at pH 9·7, in evacuated vessels for 3 hr. at 30°, produced no serious loss in cellulase activity. Likewise, hand grinding of freeze-dried powders with ballotini beads in a mortar, or grinding followed by autolysis in 0·01 % sulphide (pH 7·0) for 1 hr. at 30°, did not decrease cellulase activity. On the other hand, 0·05 % (w/v) Triton X-100 (Rohm and Haas Co., Philadelphia, U.S.A.), 0·1 % (w/v) Teepol XL (B.D.H.; Walker & Levvy, 1951) or 0·2–2 % (w/v) sodium cholate when used as solubilizing agents, all produced complete loss of cellulolytic activity. The aqueous supernatant fractions obtained by high-speed centrifugation from any of the above treatments, did not hydrolyse cellulose powder (Whatman). Likewise, acetone powders prepared from lyophilized powders and subsequently extracted with water, with 0·01 % (w/v) sulphide (pH 7·0) or with Sörensen phosphate buffer containing 0·01 % sulphide (pH 7·0), also did not give an active cellulase in the centrifuged supernatant fluid. However, some cellulolytic activity was obtained from the lyophilized powder by suspension in 0·01 % sulphide, (pH 7·0) and separation of the supernatant fluid before or after treatment with butanol, in a manner similar to that described for butanol extracts of R.P. (Methods). The aqueous butanol extract hydrolysed up to 10 % of the cellulose powder (Whatman) under the standard conditions. A sample of the aqueous supernatant fluid which was separated before treatment with butanol, was compared with the butanol extract for β-glucosidase activity on *o*-nitrophenyl-β-glucoside (by Dr J. Conchie). Approximately twice as much activity was present in the butanol extract as in the aqueous supernatant fluid. This suggests that glucosidase activity, like cellulase, is relatively closely associated with insoluble material, in agreement with Conchie (1954).

Enrichment cultures of rumen micro-organisms

In order to increase the cellulase content and to decrease the number of non-cellulolytic organisms in the concentrated suspensions of rumen liquor micro-organisms (R.P.), cultures were prepared grown on cellulose powder (Whatman). At first the standard assay conditions (see Methods) were used unmodified on the large scale. With rumen liquor itself as an inoculum (12 %, v/v) a much slower rate of cellulolysis was obtained than on the smaller scale of the standard assay. This difficulty was eventually overcome by the use of an eight-fold concentrate of rumen micro-organisms in presence of yeast extract and the clear high-speed supernatant (R.L.S.) from rumen liquor (see final medium in Methods). Where samples were required for analysis at successive periods of the fermentation, it was found advisable to use replicate vessels rather than to take successive samples from a single vessel after mixing by gentle shaking. The latter treatment invariably decreased the degree of cellulolysis to less than one-third of the value obtained by replicate sampling (Fig. 5). In some experiments, retardation of fermentation by shaking was so severe that cellulose utilization did not exceed 12 % of the available cellulose in 4 days. Such findings were in agreement with the work reported earlier on the inhibitory action of shaking on the cellulolysis of cellulose powder (Whatman) by rumen micro-organisms (Halliwell, 1957). Cellulose utilization was examined in enrichment cultures on 0·4 % (w/v), 1·2 % (w/v) and 3 % (w/v) cellulose powder (Whatman), and cellulase formation was examined on the 1·2 and 3 % cellulose powder (Whatman) suspensions (Fig. 5). Samples of fermented medium were removed at intervals from replicate vessels and analysed for cellulose and for cellulase by the standard techniques. As the results indicate, cellulase production (as determined by the standard cellulase assay) reached an optimum well before complete utilization of cellulose. When approaching the end of fermentation, as determined from cellulose utilization, some fermentations did not show any trace of cellulase when samples were removed to fresh cellulose powder (Whatman), suspensions, although residual cellulose was still being solubilized in the parent fermentation.

Three basic types of buffer salts media were used in the present investigation with enrichment cultures in order to compare their effectiveness in supporting cellulase formation: (1) the acetate salts medium of Sugden (1953) as used previously for protozoal cellulolysis (Halliwell, 1957); (2) the bicarbonate buffer medium of Bryant & Doetsch (1954) who used this medium to isolate rumen cellulolytic organisms; (3) the standard bicarbonate buffer medium of the present investigation (see preparation of enrichment cultures under 'Methods'). Fig. 6 illustrates the superiority of our standard bicarbonate medium, based on that of Elsden (1945), over the other two. Whereas the former produced rapid and active cellulolysis and cellulase formation, the alternative media gave die-away curves for cellulose utilization and for enzyme synthesis. The main difference between the three media is in the higher NaCl concentration in medium 1 and the lower bicarbonate and magnesium concentrations of media 1 and 2. As shown previously (Halliwell, 1957; Table 1) NaCl at 0·1 M (0·6 %), a concentration near that (0·5 %), of medium 1, diminished cellulolysis by rumen micro-organisms to about one-half. The same table also indicates that decrease of the magnesium concentration probably accounted for some of the inferiority of media 1 and 2 when used for cellulolysis in enrichment cultures. The main factor supporting maximum cellulolytic activity in the standard bicarbonate salts medium appeared to be its higher bicarbonate content. In this respect it was found that rumen liquor lost none of its cellulase when adjusted to pH 5·8 for 5 min. at 18° with 0·01 M-HCl and then returned to pH 6·8 for the standard cellulase assay. When, however, rumen liquor at pH 5·8 was assayed as usual, but in standard bicarbonate buffer at pH 5·8 (by using more dilute bicarbonate) instead of pH 6·8, no cellulose was hydrolysed. Whether the effect on cellulolysis is due to lowered concentration of bicarbonate or to the pH value was not

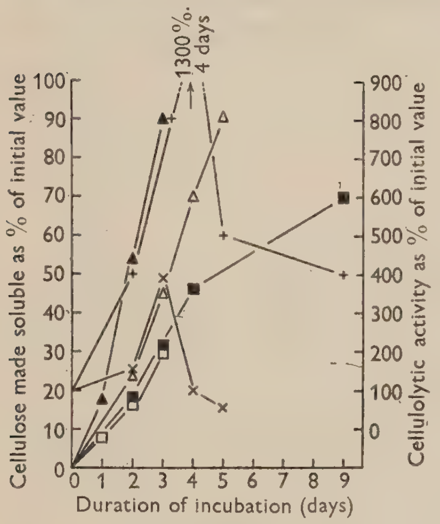

Fig. 5

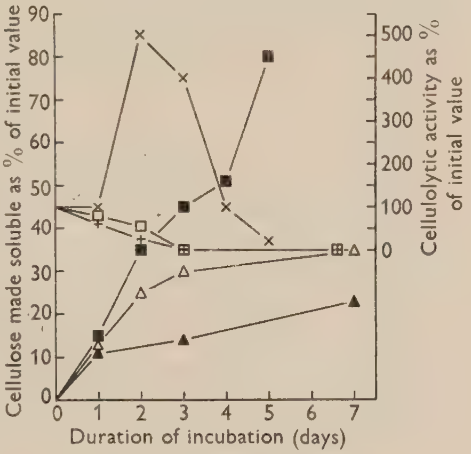

Fig. 6

Fig. 5. Enrichment cultures of rumen liquor; effect of sampling procedure on cellulolysis and effect of cellulose concentration on cellulose solubilized and cellulase produced. Cellulase determinations by standard procedure. Substrate: cellulose powder (Whatman). On 0·4 % initial cellulose: cellulose solubilized by sampling from separate vessels ▲, by sampling repeatedly from single vessel □. Results on 0·4 % cellulose for 3 days fermentation only. On 3 % initial cellulose: cellulose solubilized ■, cellulase activity +. On 1·2 % initial cellulose: cellulose solubilized △, cellulase activity ×.

Fig. 6. Enrichment cultures. Effect of different media on cellulose solubilized and cellulase activity on 1·2 % (w/v) cellulose powder (Whatman). Acetate medium (Sugden, 1953) cellulose solubilized ▲, cellulase activity +; Bryant & Doetsch medium (1954) cellulose solubilized △, cellulase activity □; bicarbonate medium (present author) cellulose solubilized ■, cellulase activity ×.

determined. However, the effect appears to be associated with bicarbonate concentration because all three media of Fig. 6 possessed approximately the same initial pH value (see Fig. 7), whilst cellulolytic activity increased as the added bicarbonate increased (from 0 % in medium 1 to 0·2 % in medium 2 and 1·5 % in medium 3). The buffer capacity of medium 3 (standard bicarbonate buffer) was much greater than the other media and was better able to resist the acidifying effect due to the metabolic products of cellulolysis (Fig. 7). Bryant & Doetsch (1954) observed a decrease in acid production by a cellulolytic organism growing on glucose when the bicarbonate of the medium was decreased from 0·2 to 0 %; they attributed this action to the bicarbonate without reporting pH changes.

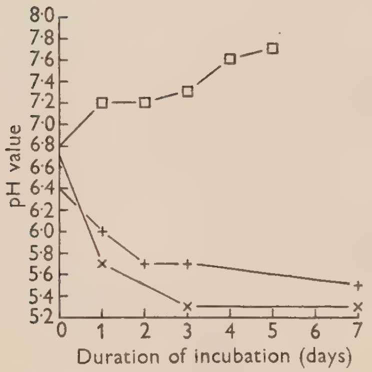

Fig. 7

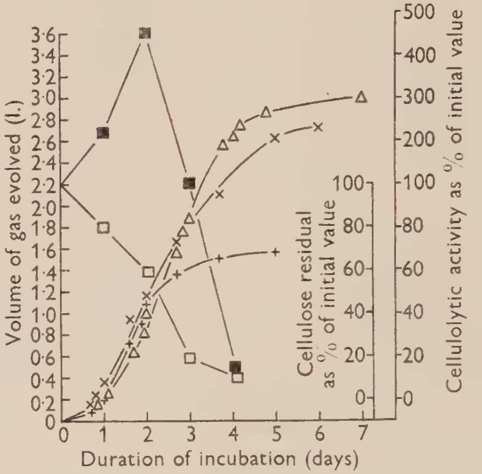

Fig. 8

Fig. 7. Enrichment cultures. Effect of different media on pH value of fermentation on 1·2 % (w/v) cellulose powder (Whatman). Acetate medium (Sugden, 1953) × ; Bryant & Doetsch medium (1954) + ; bicarbonate medium (present author) □.

Fig. 8. Enrichment cultures. Gas evolution, cellulose solubilization and cellulase activity on cellulose powder (Whatman). Gas evolution on 1·2 % (w/v) cellulose +, on 2 % cellulose △, on 3 % cellulose ×. On 1·2 % cellulose: cellulose residual □, cellulase activity ■. Cellulose residual given, not cellulose solubilized, to avoid confusion of the curves.

Determination of the course of fermentation from gas evolution

As shown above, cellulase formation appeared greatest in enrichment cultures at the early stages of fermentation, only to fall rapidly later when cellulose utilization was approaching a maximum. To facilitate the identification of the optimal time for harvesting organisms with maximum cellulase content, the fermentation vessels were connected so that issuing gases were collected and measured (see Methods). Thus, the state of metabolism (recorded in volumes of gas) was determined immediately, and after calibration against cellulose utilization and cellulase formation, provided a rapid method of following cellulolysis to the harvesting period.

Fig. 8 indicates the gas evolution over the complete fermentation cycle on cellulose powder (Whatman) at 1·2, 2 and 3 % concentration, together with the cellulose utilization and cellulase production on 1·2 % cellulose powder (Whatman). This concentration of cellulose was finally adopted for the preparation of organism extracts; the results should also be compared with those of Fig. 5. Gas evolution on 0·4 % cellulose powder (Whatman), approximately the concentration used on the small scale of the standard cellulase assay, was also examined but the curves obtained were always flatter and less decisive. Cultures growing on 1 % initital cellulose appeared most useful for obtaining concentrated preparations of cellulase-containing organisms in that residual cellulose was less troublesome when making aqueous extracts of enzyme. Maximal cellulase synthesis was found in the early stages of cellulose utilization, at about the 40 % level, just before the rate of gas evolution began to fall off.

Preparation of cellulolytic extracts from enrichment cultures of rumen micro-organisms

A concentrated precipitate of cellulase-containing micro-organisms obtained from the above enrichment cultures by high-speed centrifugation was used to prepare alumina-ground extracts (see Methods). Under optimal conditions the enzyme preparation so obtained was capable of solubilizing up to 30 % of the cellulose powder (Whatman) supplied, but did not hydrolyse de-waxed cotton fibres. Maximum cellulolytic activity was obtained by grinding and extracting the organisms with alumina at pH 7·2 rather than at pH 6·5; cellulase action appeared to be associated with yellow-coloured extracts which were more readily obtained at higher pH values. Corresponding alumina extracts from concentrated precipitates of non-enrichment cultures did not yield cellulase in the extracts. The orange coloured butanol extracts prepared from enrichment cultures like those directly from concentrates of rumen micro-organisms (non-enriched), never hydrolysed more than *c.* 10 % of the cellulose powder (Whatman) in 2 days at pH 6·8 (standard bicarbonate assay buffer) or at pH 6·0 (in Sörensen phosphate buffer). As shown above (Fig. 3), rumen butanol extracts were more effective against the soluble cellulose derivative carboxymethylcellulose.

Comparison of the cellulolytic activity of the various preparations

It was also found that the cell-free filtrate (see Methods) obtained from *Myrothecium verrucaria* cultures growing on cellulose powder (Whatman) had properties very similar to those of the alumina-ground extracts of rumen micro-organisms from enrichment cultures. Thus, the extracts from concentrates of rumen micro-organisms solubilized about 30 % of cellulose powder (Whatman) whilst our best filtrates (unconcentrated) from *M. verrucaria* were equally active in the same period (72 hr). Likewise, the rumen alumina-extract and the *M. verrucaria* cell-free filtrates had only feeble activity (in contrast to the butanol preparations from rumen micro-organisms) against carboxymethylcellulose. Thus the enzyme activity ratios of a butanol extract (from a concentrated preparation of micro-organisms) and of a cell-free filtrate (unconcentrated) from *M. verrucaria*, were approx. 5:1 at 37° with carboxymethylcellulose as substrate (assays by courtesy of Dr G. N. Festenstein). On the other hand, with insoluble cellulose as substrate, the butanol extract (rumen) possessed only one-half the activity on cellulose powder (Whatman) and one-fifth the activity on swollen cellulose powder, of the *M. verrucaria* filtrate. Table 2 shows some of the properties of *M. verrucaria* filtrates on these substrates. The table also indicates that the active principle in *M. verrucaria* filtrates towards cellulose powder was unstable at higher temperatures.

Table 2. *Action of* Myrothecium verrucaria *cell-free filtrate at (pH* 6·0) *in Sörensen phosphate buffer*

Standard *M. verrucaria* cellulase assay (see Methods). C.P.W. = cellulose powder (Whatman); S.C.P.W. = swollen C.P.W.

		30°		50°
		Static tubes	Shaken flasks	Static tubes
	Period of	Cellulose used as % of initial		
Substrate	incubation (hr.)			
C.P.W.	48	19	20	8
S.C.P.W.	18	—	75	88

DISCUSSION

In a previous paper (Halliwell, 1957) it was emphasised that the results of work on cellulolysis by micro-organisms or extracts thereof, depend not only on the substrate used but also on the method of cellulase assay employed. This theme was then developed to analyse the validity of results obtained by different methods of assay, particularly those which depend on formation of reducing sugars from insoluble forms of cellulose. Different forms of cellulose contain chains of very different length, ranging from the complex native cotton fibre down to the much-degraded cellodextrins and the soluble but substituted cellulose derivative carboxymethylcellulose. Certain micro-organisms can produce complete solubilization of all these forms of cellulose and, as shown, rumen micro-organsims are one of the most active sources known. Many workers have prepared extracts, from animal and plant sources and from cellulolytic and non-cellulolytic micro-organisms, which rapidly hydrolyse soluble, but substituted, cellulose derivatives, and have little or no action on insoluble cellulose, particularly cotton fibres. Recent examples of such preparations have been collected (Halliwell, 1957) and calculation shows that the degree of hydrolysis ranged from 0·2 % to about 6 % of the available cellulose powder or cotton. Reese, Siu & Levinson (1950) explained this difference by suggesting that action on insoluble cellulose involves a preliminary cellulase (C_1), sparsely distributed in nature, followed by the action of a more universal enzyme (C_x). The latter is also believed to hydrolyse the much degraded soluble forms of cellulose or cellulose derivatives. The low degrees of hydrolysis of insoluble cellulose obtained by these workers (0·2–6 % solubilization) may well represent enzymic breakdown of non-reducing, non-true cellulosic fractions (xylans, glucosans) associated with relatively undegraded cellulose. Hydrolysis of such 'impurities' by a 'cellulase' preparation, when analysed by a reductimetric method would yield false evidence of cellulolysis. The error assumes major importance when the observed degree of 'cellulolysis' represents only a fraction of the available cellulose. Support for this belief of 'impurities' being present is to be found in the identification of xylose from a fermentation of cellulose powder (Alphacel) by rumen micro-organisms (Kitts & Underkofler, 1954) and of the short-chained polysaccharides obtained by aqueous extraction from filter-paper, wood cellulose and cotton linters (Huffman, Rabers, Spriesterbach & Smith, 1955). An example of this low degree of cellulolysis of insoluble cellulose found by a reductimetric method of cellulase assay is given by the work of Kitts & Underkofler (1954), who obtained a preparation from rumen bacteria which produced only 3 % cellulolysis of cellulose powder (Alphacel) in 49 hr.; further example appears to be the preparation from *Myrothecium verrucaria* obtained by Whitaker (1953). After concentration and purification of *M. verrucaria* filtrates, the latter hydrolysed no more than 2 % of the weight of cotton linters and no more than 7 % of a 'cellulose' precipitated from cotton dissolved in concentrated sulphuric acid. Thus, hydrolysis of the cotton linters was low, possibly due to the few susceptible short chains, whilst hydrolysis of the acid-degraded cellulose, with more of the shorter chains, was correspondingly greater. Whitaker's cellulase may have been exhibiting uni-enzymic action, but only towards the shorter molecular species of cellulose chains present in most forms of insoluble cellulose.

Our own findings tend to support the views expressed by Reese *et al.* (1950) that more than one enzyme (C_1 and C_x) or set of conditions may be necessary for significant hydrolysis of insoluble cellulose. Thus, our rumen butanol preparations acted much more effectively against the soluble carboxymethylcellulose, but had only slight hydrolytic action (maximum 10 % hydrolysis) on insoluble cellulose powder. On the other hand, alumina extracts from rumen micro-organisms and cell-free filtrates from *Myrothecium verrucaria* were relatively feeble in attacking carboxymethylcellulose as compared with the rumen butanol extract, but produced up to 30 % solubilization of insoluble cellulose powder. Such a reversal of enzyme activity on a soluble cellulose derivative and on insoluble cellulose suggests multi-enzymic action (or conditions). A similar variation of activity in filtrates of other organisms was reported by Gilligan & Reese (1954). Further evidence for the dissimilarity of the enzyme which hydrolyses carboxymethylcellulose from that which hydrolyses insoluble cellulose appears in the work by Reese *et al.* (1950) who showed that *M. verrucaria* filtrates acting on soluble carboxymethylcellulose were about twice as active at 50° as compared with 30°. In contrast, with an insoluble cellulose powder (Whatman) our own work indicates that *M. verrucaria* filtrates exhibited less than half their activity when incubated at 50°, as compared with their effect at 30°.

The nature of the initial action on insoluble cellulose is still uncertain. If it be enzymic in nature, our results suggest 'the enzyme' may be highly unstable or requires a special combination of conditions for (1) no true cellulase was detected in the medium external to rumen micro-organisms growing on insoluble cellulose, (2) true cellulase activity of rumen micro-organisms fermenting insoluble cellulose powder was severely repressed by the presence of antiseptics (Halliwell, 1957) as compared with their action on the soluble carboxymethylcellulose, (3) exposure of cellulase-active rumen micro-organisms, of freeze-dried powders or of acetone powders of rumen organisms, to excessive aeration or elevated temperatures produced heavy losses of cellulase and (4) no one has yet succeeded in preparing cellulolytic extracts which attack undegraded cotton fibres with anything remotely approaching the vigour of micro-organisms.

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REFERENCES

Bentley, O. G., Lehmkul, A., Johnson, R. R., Herschberger, T. V. & Moxon, A. L. (1954). The 'Cellulolytic factor' activity of certain short chained fatty acids. *J. Amer. chem. Soc.* **76**, 5000.

Bryant, M. P. & Doetsch, R. N. (1955). Factors necessary for the growth of *Bacteroides succinogenes* in the volatile acid fraction of rumen fluid. J. *Dairy Sci.* **38**, 340.

Bryant, M. P. & Doetsch, R. N. (1954). A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. *J. Dairy Sci.* **37**, 1176.

Conchie, J. (1954). β-Glucosidase from rumen liquor. Preparation, assay and kinetics of action. *Biochem. J.* **58**, 552.

Elsden, S. R. (1945). The fermentation of carbohydrates in the rumen of sheep. *J. exp. Biol.* **22**, 51.

Folin, O. & Malmros, H. (1929). An improved form of Folin's micro method for blood sugar determinations. *J. biol. Chem.* **83**, 115.

Gilligan, W. & Reese, E. T. (1954). Evidence for multiple components in microbial cellulases. *Canad. J. Microbiol.* **1**, 90.

Halliwell, G. (1957). Cellulolysis by rumen micro-organisms. *J. gen. Microbiol.* **17**, 153.

Huffman, G. W., Rabers, P. A., Spriesterbach, D. R. & Smith, F. (1955). Nature of a hemicellulose extracted from cellulose with water. *Nature, Lond.* **175**, 990.

Kitts, W. D. & Underkofler, L. A. (1954). Digestion by rumen micro-organisms. Hydrolytic products of cellulose and the cellulolytic enzymes. *J. agric. Food. Chem.* **2**, 639.

McIlwain, H. (1948). Preparation of cell-free bacterial extracts with powdered alumina. *J. gen. Microbiol.* **2**, 288.

Mickle, H. (1948). Tissue disintegrator. *J. R. micr. Soc.* **68**, 10.

Morton, R. K. (1950). Separation and purification of enzymes associated with insoluble particles. *Nature, Lond.* **166**, 1092.

Reese, E. T., Siu, R. G. H. & Levinson, H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J. Bact.* **59**, 485.

Saunders, P. R., Siu, R. G. H. & Genest, R. N. (1948). A cellulolytic enzyme preparation from *Myrothecium verrucaria*. *J. biol. Chem.* **174**, 697.

Sijpesteijn, A. K. & Elsden, S. R. (1952). The metabolism of succinic acid in the rumen of sheep. *Biochem. J.* **52**, 41.

SIU, R. G. H. (1951). *Microbial Decomposition of Cellulose.* New York: Reinhold Publ. Corporation.

SPURLIN, H. M. (1939). Arrangement of substituents in cellulose derivatives. *J. Amer. chem. Soc.* **61**, 2222.

SUGDEN, B. (1953). The cultivation and metabolism of oligotrich protozoa from the sheep's rumen. *J. gen. Microbiol.* **9**, 44.

WALKER, P. G. & LEVVY, G. A. (1951). The preparation and properties of β-glucuronidase. 2. Centrifugal fractionation of water homogenates and the nature of the sedimentable fraction. *Biochem. J.* **49**, 620.

WHITAKER, D. R. (1953). Purification of *Myrothecium verrucaria* cellulase. *Arch. biochem. Biophys.* **43**, 253.

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Some Thoughts on Bacterial Classification

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SUMMARY: Scientific classification is virtually a branch of mathematics which describes the overall similarities of organisms. Catalogues do not do this. Many schemes of bacterial taxonomy are not classifications but catalogues. Similarity is best measured by the number of features in common between two strains, while division into taxa is based on correlated features. Other criteria for these two basic ideas are unsatisfactory and cause confusion, and there seems to be no logical reason why any one feature should be given greater weight in classification than any other. Hierarchical systems are a practical necessity, and simple mathematical methods are useful in bacterial classification. It is not necessary to know the evolutionary history of organisms in order to classify them in a scientific manner.

It might perhaps be best if this paper were given the subheading of 'Issued but not published' following the distinguished precedent of *An Unofficial Interpretation of the International Rules of Zoological Nomenclature* (1955). But whether published or not, bacterial classification is in such confusion that it seems doubtful whether any nonsense can make it much worse than it is. These reflexions are an attempt to re-think the logic of bacterial classification. They present one point of view, perhaps carried to an extreme. None of the ideas is new; they are implicit in the work of many bacteriologists (e.g. Winslow & Rogers, 1906; Levine, 1918; Topley & Wilson, 1929; White, 1937) and particularly in the important and prophetic paper of Rogers, Clark & Davis (1914) which has been generally overlooked. These ideas underlie much of the matter read at the Discussion Meeting of the Society for General Microbiology (1954). They have long been discussed in the taxonomy of higher organisms (see Conclusions). Although in devising a particular taxonomic scheme some balance must be struck between the ideal and the practical, it is ideal scientific classifications which are considered in this paper, with an attempt to define the lines along which ideal systems might be sought. Scientific classification is used here in the sense of the 'natural' classifications of Bather (1927) or the 'general' classifications of Turrill (1952), indicating orthodox taxonomy; while the term catalogue approximates to Bather's 'artificial' classifications and to Turrill's 'special' classifications.

A PHILOSOPHY OF CLASSIFICATION

A classification is greatly influenced by the purpose for which it is devised. It may sometimes be useful to classify wild flowers by their colour—if one is teaching school children—but no one would submit this as a scientific classification. What are the principles which make us say that such a scheme is not scientific? We may first consider two purposes of classification in general:

(i) to give names or numbers to things, which is better called enumeration or cataloguing; (ii) to indicate similarity, and thus to increase our ability to think about and to use our observational material.

The giving of numbers to strains or to antigenic types is enumeration. It is questionable whether this is classification, or if so whether it is usually scientific (Cowan, 1955). It has, of course, great utility. Thus, if scientific classification were abolished, workers on food poisoning would immediately give letters or numbers to the big groups of bacteria which concerned them; group A for *Clostridium botulinum* and so on. They would classify strains by placing them into these groups, but the groups themselves would be enumerated—a catalogue. The groups would only be classified when someone pointed out that group A strains were closely related to group F (*Clostridium welchii*) but not to group B (*Salmonella*).

The underlying assumption of scientific classification is that there is a natural order, a system of similarities, which can be discovered by investigation. In its simplest form it says that A is very similar to B but is less similar to C. The distinctive concept of scientific classification is that it attempts to measure overall similarity; its distinctive value is in prediction. For example, we classify a mouse as a mammal because we believe it has sufficient overall similarity to other creatures which we already call mammals. The value of this course is that we can predict many features of any creature which is called a mammal. Other classifications do not contain such additional implied information; a classification of vertebrates into male and female, long and short, black and white, tells us nothing more, and it would never be proposed as a scientific classification. The overall similarity is measured by the number of similar features.

This assumption may be taken as axiomatic but it has a logical basis. Theoretically every feature would seem to be of equal weight for estimating overall similarity and for the following reasons. (1) Unless we prove that any particular feature should carry greater weight than another we must either give equal weight to every feature or else give weight on unproven or irrational grounds such as giving weight to features whose initial letter is A. (2) When we do consider that a feature has greater weight than another it is because it is correlated with other features, or because we believe that it 'sums up' a number of unknown features, which also implies correlation. (3) The greater weight is thus derived from correlation, and if we find an exceptional individual the feature is here no longer correlated with its usual congeners and, for classifying that individual, it loses its heavier value. Weighting is thus inseparable from correlation (and this also implies division). It is not the concept of number which is difficult but how one should estimate the number (see Conclusions).

The operative word here is number. Overall similarity is the concept of the sharing of many features, not of any one particular feature. Thus in Leviticus (*Lev.* xi. 19) the bat is catalogued with the birds because it can fly; the modern zoologist classifies it as a mammal, and his reasons are convincing, since the bat resembles other mammals in many features and resembles the birds in but few. This concept does not consider evolution but it is not invalid on that account (see Conclusions).

Catalogues. The trouble with catalogues is that they are endless, arguable and of poor predictive value. They appear precise, but are vague, while paradoxically good classifications may appear vague but are precise. No one can prove that one catalogue is any 'truer to nature' than another. The enumerator is soon driven to defining 'groups' and to predicting other features of new strains because they fall into one of his groups. Before long he is back at devising schemes of similarity. For practical purposes, therefore, since we cannot easily think about catalogues or remember them, we are driven back to classification.

On similarity and division

There is a distinction to be made at this point between expressing similarity and dividing groups of organisms. A series of strains may vary in the amount of an enzyme which they produce, and this can be expressed in mathematical terms if one wishes, giving a set of figures which are a measure of similarity in this respect. When we start to split up this series of strains our aim is to group the infinite array of possible figures so that we can conveniently think of them. Now a classification can in theory be made by listing similarities without any attempt to separate the strains into taxonomic groups but in practice we need some device for compressing the information, which is why we use hierarchical systems of classification, with different ranks of taxa to indicate roughly the degree of similarity. God can consider every creature as an individual—we have to group them into such things as genera and species. These are artificial concepts; they have no actual existence. Only creatures exist (Adanson, 1763, Préf. p. clxiii); Pirie (1955) has well summed this up.

Separation. We are faced with the practical necessity of separating groups of organisms. How shall we do so? We may first consider the position of higher organisms. The history of classification abundantly shows the uncertainty of intuition in deciding what features are 'important' in classification (witness bats and whales) yet we all know of features that are of little importance. Let us consider how one separates two groups, for instance cats from dogs. There are features which we know are very variable and unimportant in such a separation, such as colour. We call them 'unessential' features. But how do we tell 'essential' features? No single feature will do. If one found a dog which had a cat's incisor teeth, it would indeed be a surprise! But this would not make the creature a cat, for we would thereafter relegate it to the 'unessential' features. The reason for regarding a feature as 'essential' is simply that it *always goes with some other feature.* Consider the position if this were not so, and if there were all possible combinations of the various features of cats and dogs: we should be unable to separate the two kinds of animal because there would in fact be no dividing line. This is associated in higher organisms with genetic exchange, such as the interbreeding of the breeds of dog. In bacteria there is probably a great deal more genetic exchange than we are aware of at present, which may be one reason why we often find intermediate forms between bacterial 'species'.

In higher organisms we also find intermediate forms, but this does not shake our faith in classification (that is to say *degrees of similarity*). It is simply an expression of the fact that relationship is an infinitely variable quantity. Our divisions are those of convenience. It is obvious that we place the lines of division where the resultant groups will hold the greatest content of information. There are 'points of rarity' in distributions of features where individuals with certain combinations of features are rare (for instance the platypus is close to the point of rarity between mammals, birds and reptiles) and since we are studying the world as it is we will place the divisions at the points of rarity. No one wishes to classify non-existent organisms, that is to say, those which are infinitely rare. This course is also the convenient one, for in order to think about groups of bacteria, to make mental concepts, to arrange groups, taxa, species or what not, we are forced for reasons of mental economy to consider chiefly the commoner forms. If there is no point of rarity, it is useless to make a division, since we say 'these cannot be separated'. Indeed they can be separated, and sometimes are, in a purely arbitrary fashion, as when we divide cats into white cats, brown cats and so on, but this is not a measure of the *overall* similarity between various cats, and may, by its very arbitrariness, obscure real relationships of a subspecific kind.

This leads to the concept of a taxon as a group of strains with many features in common but not necessarily always possessing any particular feature—what may well be called a 'species-group' or a biotype. Unfortunately these terms have other meanings; e.g. a biotype is defined, in the International Bacteriological Code of Nomenclature (1948) Recommendation 8*a* (2), as a physiological type for which physiotype might be a better word but for which no other suitable term seems available; and it seems to be necessary to create new names for the kind of groupings found when considering bacteria. Existing terms based on the degree of interbreeding (see Turrill, 1952) are not suitable. It is desirable to distinguish between the reactions of strains and of groups of strains. The ending *-form* is suggested for the former, and the ending *-on* (as an abbreviation of taxon) for the latter. The following situation, frequent in bacteria, may be envisaged. Two groups of strains occur (the strains within each group being very similar to one another), and each group might be termed a *pleiston* (from the Greek πλεῖστος, very many). The most common type within a pleiston—perhaps only a hypothetical strain—would be the *modoform* of the pleiston, and the other strains composing the pleiston might be called *sychnoforms* (from the Greek συχνός, frequent). The groups are connected by uncommon intermediate forms (*interforms*), and this connecting group if it needed a separate name might be termed a *gephyran* (from the Greek γέφυρα, a bridge). There may also be less common groups of strains clustered around the pleistons and connected to them by interforms: such groups might be called *oligons* (from the Greek ὀλίγος, scanty). The oligons are each composed of a modoform and of sychnoforms. A good example is the *Bacillus cereus–Bacillus megaterium* group (Smith, Gordon & Clark, 1946). The typical strains of *B. cereus* would form one pleiston and the typical strains of *B. megaterium* would form another pleiston, the *B. cereus–B. megaterium* intermediates would be interforms, while anthrax bacilli would form an oligon attached to the *B. cereus* pleiston. Although further work may lead to better separation of the organisms given in this example, the scheme is useful at a certain stage of knowledge. As Smith, Gordon & Clark (1952) remark, the Rules of Nomenclature do not adequately cover situations such as this. For instance, if the anthrax bacillus is regarded as a variety of *B. cereus*, according to the rule of priority the former should be named *B. anthracis* var. *anthracis* and the latter *B. anthracis* var. *cereus*. The two criteria used for defining these new terms are the degree of similarity and the commonness of the strains. These terms are not intended to have any taxonomic rank, since this is the reason for coining them instead of using terms such as species and variety. We may conjecture that this situation is found at a low level in classification, at species and genus level, and may be likened to the occasional leaves on the evolutionary tree which are partly fused to an adjoining leaf.

The alternative is to define a taxon as a group whose members all possess a specified key feature. Such features should be definable, recognizable and rigidly employed, yet they can neither be defined nor recognized before creating the taxon, and they are not in any event rigidly employed. If, for instance, the mammary gland is taken as the key feature of the mammals, how does one define it or recognize it before first making the taxon Mammalia, and is a mouse with congenital absence of mammary glands to be excluded from the Mammalia? A key feature is therefore not a feature for creating a classification, but is one which is useful for easy identification afterwards. There would seem, therefore, to be only one way in which we can decide if a feature is 'important' and that is by seeing whether it goes closely with other features.

Let us examine in bacteria some consequences of the principle of Division by Correlated Features. I do not know whether it has ever been explicitly formulated in bacteriology, though Rogers *et al.* (1914) came close to doing so. The trouble with any other method is how to decide on the relative 'importance' of features. The importance is composed of two interdependent things—the usefulness for making a division and the content of information in the divisions thus made. We may consider a hypothetical genus of bacteria which is studied by four successive authors. If the first author divides a genus arbitrarily on nitrate reduction and on lactose fermentation then every strain will go into one of the four 'species' so formed. Of course, all sorts of bacteria can be fitted in, and not infrequently all sorts of bacteria are. The second author divides the genus on reactions with maltose and with citrate. Who is to decide which is the right (or, not to beg the question, the better) classification? Is the first author to be followed because he is first?—or the author who uses the commoner tests?—or the one who uses tests in use in a nearby genus?—or the one who follows the text-book which we prefer?—or the one who uses a feature of economic as opposed to academic importance? The third author proposes eight tests and makes 256 'species'; we feel that this is too much. Which tests shall we discard and on what grounds? Now if all these tests are 'unimportant' tests (on a par with coat colour in cats and dogs) then these questions are insoluble because these tests are in fact useless for dividing the genus. The disagreements spring from the assumption that the tests must be important, but no proof is offered for this assumption. This is but fighting with shadows. Such a system—really a catalogue—not only fails to show up the relationships clearly, but may conceal them. It is to react like the Eskimo who comes to Britain and decides that white cats and sheep are polar bears. To follow our hypothetical genus, the fourth author devises some new tests J, K, L, M, N and finds that all strains are J+K+L+M−N− or are J−K−L−M+N+, that all pathogenic strains show the first set of reactions and are natural inhabitants of the bowel, and he achieves two things. He shows that the genus is separable into species, and he compresses into his classification much information of a useful kind; he has separated cats from dogs. Division by correlated features is also the best solution to the problem of the classification of intermediate forms, since if this method fails, then any arbitrary method will also fail.

The Enterobacteriaceae

A notable example of the difficulty in classifying a group of bacteria is the Enterobacteriaceae. These organisms show a bewildering array of features in almost all combinations, so that they cannot be easily and neatly divided. Kauffmann, Edwards & Ewing (1956) somewhat pessimistically reject the Linnaean hierarchy, apparently because it is Linnaean, not because it is hierarchical. But does anyone now accept Linnaean concepts of species, and is this not a different thing from Linnaean binomial monenclature? If a scheme be rejected because it is hierarchical, then one has tacitly admitted that strains cannot be grouped into taxa of larger size. The above authors, however, admit that they have to use various groupings for practical reasons. They abandon the 'artificial and arbitrary classification into "genera" and "species"' and instead use biochemical groups, subgroups and types. In what way are the latter any less artificial or arbitrary? Or are the other difficulties they mention the reason for this change? Their main point seems to be summed up in the sentence 'under these circumstances...it is impossible to decide whether a given classification is scientifically correct, but only whether it is more or less practical and expedient'. Although it is not clear to me just what they mean by 'scientifically correct', this is at any rate a candid admission of our poor knowledge, and is far better than creating multitudes of bad taxonomic groups. The Enterobacteriaceae can only be better classified when more work is done on correlated features and the frequency of intermediate forms. It seems probable (as has happened in other groups which are largely parasitic, e.g. some fungi—see Ainsworth, 1955) that in the past we have in the Enterobacteriaceae made our species too small (Cowan, 1956*a*); we may be trying to separate the inseparable.

PRACTICAL ASPECTS OF CLASSIFICATION

Avoidance of preconceived notions. When collecting strains for study we should not be too selective, or too obsessed with the best tests to use. With all of us there is a tendency to exclude a strain because it differs in a 'cardinal' feature. There is little interest and no significance in a statement such as 'All streptococci are Gram-positive' if every Gram-negative organism is resolutely discarded. Some limit must be set but it is better to cast the net widely, and to exclude on several features, rather than risk discarding an aberrant streptococcus which is Gram-negative. Some taxonomists appear to exhibit strong intuitions about the value of certain tests, but it is difficult to think of any test which is invariably reliable in the separation of bacteria. The Gram reaction, flagellation, carbohydrate metabolism, pathogenicity, aerobiosis, anaerobiosis, antigenic structure—all these have proved to be unreliable in some genus or another, either because genuine exceptions occur, or because the conditions of testing are so critical that in practice one gets false results. It is well to be sceptical about well-established tests even in groups where they have proved their value; the proof may not be very sound.

Definition of taxa and dichotomous keys

If we accept the importance of divisions based on correlated features, what happens when we summarize our classification in order to define our taxa? We will use in our generic definition those features which are common to all strains. Of the other features we will obviously choose those with high correlation for dividing the genus into species, thus:

Genus is $A+B+C-D-$

Species 1 is $E+F+G-H-$

Species 2 is $E-F-G+H+$

To indicate the range of variability, we will add 'tests I, J, K, L variable in both species'. A common complaint is that generic definitions contain features qualified by the word 'usually'. There is some substance in such complaints, since this implies that the features listed are not well correlated, but from the theoretical point of view, every feature must be so qualified. If enough well-correlated features are used, the description will be adequate. One improvement on the usual way of listing features may be suggested: features can be given in two sections, (i) those which are almost always found and (ii) those which are less constant. The problem becomes most acute when we try to make the dichotomous keys so beloved of text-books. Shall we use more than one feature? If we use one feature, then the exceptional strain will be misclassified: if we use several features the exceptional strain will not fit in and the key may become unwieldy.

Single-feature keys are pernicious since they suggest that the group can be sharply divided. If one must have a key it is better to use several features, since the user can then see that his strain is aberrant in a single test, and it is possible that he will make the logical deduction that he has an aberrant strain. Single feature keys look neat, but the user thinks he has made a mistake (which he may have done) or that he has a new species (he is probably wrong), or else (and this time he is right) that the key is nonsense. The formal logical way is to use several features for the key and to add 'intermediate forms may occur'. Keys which are qualified by exceptions to cover likely difficulties presuppose a thorough knowledge of the likely aberrancies. The most successful general key of this kind is that of Skerman (1949).

Conditions of test. One practical difficulty is always arising, and that is the standardization of tests. This may be illustrated by the example of growth temperatures. If we have two species with different optimum temperatures should we do all our tests at one temperature, or test each species at its optimum? Our chief difficulty is that we do not know how growth at non-optimal temperatures will affect the results and we have not the time to investigate this. Individual strains will also have different optima. I feel that our practice should be devised with an eye for the future worker. He cannot repeat the tests unless the instructions are precise, and he may be too lazy to find out the optimum temperature for each strain and to marshal an array of incubators. It is best to use one temperature for both species, for the risk of all our tests being vitiated seems less than the risk of making our work unrepeatable. Cowan (1956*a*) recently made a forceful plea for standardization of descriptions and reform of nomenclature.

The use of mathematics in devising a plan of work

When one studies a group of bacteria one naturally begins by collecting strains and listing the tests to be used. Commonly one is faced with the dilemma—shall I do a lot of tests with a few strains, or a few tests with many strains? Some help may be obtained from a very elementary consideration of probability theory. We wish to find how to separate a group into two or more species, and make these assumptions:

(1) The majority of our strains belong to one species (Species 1) but we have in the batch of strains which we pick for testing at least two strains which belong to a different species (Species 2). At this stage we cannot tell which strains are Species 2, and the probability that a strain picked at random from those available will belong to Species 2 may be called p_s.

(2) We must discover at least two tests which show absolute correlation in dividing the two species, for example Species 1 is A + B + and Species 2 is A − B −, but no strain is A + B − or A − B +. The probability that any test picked at random from available tests will prove a 'good' test in this sense, may be called p_t.

We may now ask what are the chances that we shall 'hit the jackpot'—i.e. discover that there are two or more strains of the second species which are separable on two or more 'good' tests. This problem is a variation of the problem of finding two or more successes in a random sample of n trials when the possibility of success per trial is p. The required probabilities can be obtained from the binomial expansion, and some of them are tabulated in Table 1. In Table 1 p can stand for p_s and n for the number of strains chosen (giving the chance of including two or more Species 2 strains), or else p can stand for p_t and n for the number of tests employed (giving the chance of finding two or more 'good' tests). To solve our problem we must succeed in both of these respects, and the overall chance will be the product of the chances of each individual eventuality. An example may be given: we are sent a thousand strains of streptococci, which are mostly enterococci, but twenty are group A streptococci. We pick one hundred strains at random, and therefore $n=100$ and $p_s=0\cdot 02$, and the chance of having two or more group A streptococci in this selection of a hundred strains is 0·60 (ignoring the 'two-tailed' distribution of P_s). We have a hundred tests available, and of these five are 'good' tests, so that $p_t=0\cdot 05$. We choose fifty tests at random, and the chance of picking two or more good tests is 0·72. We test the hundred strains in the fifty tests: the chance of showing that there are two kinds of streptococcus on these results will be $0\cdot 60\times 0\cdot 72=0\cdot 43$, about an even chance.

Table 1. *The probability of finding two or more successes in n independent trials when the probability of success in a single trial is p*

The values are obtained from the expansion of the binomial $(p+q)^n=1$, and are equal to $1-(q^n+npq^{n-1})$.

		p					
		0·5	0·2	0·1	0·05	0·02	0·01
n	2	0·25	0·04	0·01	0·00	0·00	0·00
	5	0·81	0·26	0·08	0·02	0·00	0·00
	10	0·99	0·62	0·26	0·09	0·02	0·00
	20	1·00	0·93	0·61	0·26	0·06	0·02
	50	1·00	1·00	0·97	0·72	0·26	0·09
	100	1·00	1·00	1·00	0·96	0·60	0·26
	200	1·00	1·00	1·00	1·00	0·91	0·59
	500	1·00	1·00	1·00	1·00	1·00	0·96
	1000	1·00	1·00	1·00	1·00	1·00	1·00

Table 2. *The probabilities of 'hitting the jackpot' are given under the headings* (*a*), (*b*), *and* (*c*), *where* (*a*) *is* 10 *strains in* 100 *tests*, (*b*) *is* 32 *strains in* 32 *tests*, (*c*) *is* 100 *strains in* 10 *tests for various values of* p_s *and* p_t

p_s	p_t	(*a*)	(*b*)	(*c*)
0·5	0·5	0·99	1·00	0·99
0·5	0·1	0·99	0·84	0·26
0·5	0·05	0·95	0·48	0·09
0·1	0·5	0·26	0·84	0·99
0·1	0·1	0·26	0·71	0·26
0·1	0·05	0·25	0·40	0·09
0·05	0·5	0·09	0·48	0·95
0·05	0·1	0·09	0·40	0·25
0·05	0·05	0·09	0·23	0·09

If the values of p_s and p_t are both 0·5 we shall quickly get the answer, while if either is less than 0·01 we are unlikely to do so without hundreds of trials. The practical range will therefore be 0·5 to 0·01. Here is an example. We decide to use only 1000 culture tubes, and can do (*a*) 10 strains in 100 tests; (*b*) about 32 strains in 32 tests; (*c*) 100 strains in 10 tests. The probabilities that we will 'hit the jackpot', though they vary with p_s and p_t, are usually in favour of the second course (*b*). In Table 2 are given the probabilities from this example for some values of p_s and p_t. It is seen that course (*a*) or course (*c*) is seldom very much better than course (*b*), while the superiority of course (*b*) is very evident when both p_s and p_t are small.

If we knew the value for p_s or p_t we should know what to do without the aid of mathematics, but if we do not, then it seems wisest to choose the middle course (*b*). The example given is greatly over-simplified, but the general statistical conclusions would seem to hold for more complex instances, particularly if we wish to find three or more species (or 'good' tests), when the individual probabilities become smaller for given values of p_s or p_t.

We may therefore conclude that the answer to our dilemma is to choose a middle course. This is at variance with the teachings of those who emphasize the importance of using many strains. The importance of using enough tests is emphasized here, if need be by decreasing the number of strains. There is no shortage of possible tests; Clarke (1955) has suggested ways of devising a great many new tests. Another advantage of numerous tests may be mentioned. If we use many tests we will find more 'good' tests, and our division will be more firmly established—it is better to have two firmly established species than to find (by using many strains) four or five poorly established species. There are enough of the latter already cluttering the literature.

The use of mathematics in testing the significance of our findings

We start with the two assumptions made previously, namely, that we have at least two strains of Species 2 which is separated on two or more completely correlated tests. We make out a large table, with strains down the side and tests along the top and fill in the results. We see a pattern which suggests we have 'hit the jackpot'! Is this pattern due to chance? We might have chosen strains and tests which by chance gave the pattern we see. It is obvious that if we used four strains and two tests we could not place much reliance on the assertion that species 1 was A+B+ and species 2 was A−B−. If, however, in 100 strains we had 50 A+B+ and 50 A−B− strains it would be superfluous to do a test of significance. The best method of showing one's results is to publish the full table of all the results with every strain. Editors, wishing to save space, will object to this; they should be resisted if possible, for if we condense our table we may lose vital information—literally vital in the sense that our classification will live or die by the information we retain. It is for example very difficult to analyse the massive data on the Enterobacteriaceae, since the reactions of individual strains are usually not given. Most of the relevant information will be preserved if we use correlated features for our classification, but there may be imperfectly correlated data which are of great importance.

One way of condensing the table, which discards little information (and is a useful way of preparing the information for statistical analysis) is to take a pair of tests and count the numbers of strains positive in both, negative in both and positive with the first but not the second and vice versa, and arrange them in a square to give a 2×2 table—e.g. for 92 strains we may have:

		Test A	
		+	−
Test B	+	40	0
	−	1	51

This is repeated for each way of pairing-off the tests, and arranged in a larger table as shown below. The last tests (A in the rows and E in the columns) are redundant and are omitted. The triangle gives all the possible ways of pairing the tests. It is a saving of space, and can accommodate the results of any number of strains. One can easily see the degree of correlation between tests, and calculate the probabilities where necessary. A further saving, though it causes loss of more information, is to omit the numbers of strains from such a table, and enter only the probabilities against the distribution in each square being due to chance. Alternatively, the information can be given in a form rather like this: 'Tables of contingency were made for all possible pairs of tests and the probabilities that the associations were due to chance were calculated. None showed values below 0·05 except for the pairs AB ($P=0{\cdot}001$), AC ($P=0{\cdot}01$) and BC ($P=0{\cdot}02$).' The most logical method, to tabulate the number of similarities and differences between strains, is scarcely suited for publication, although it is the way in which the data would be analysed in a calculating machine, as is mentioned later.

	A	B	C	D
E				
D				
C				
B				

The probabilities are *not* a measure of how close the correlation is, for an absolute correlation based on only a few strains may have the same probability as an imperfect correlation based on a large number of strains. We lose this information in any event. The probability is a measure of whether we are entitled on our results to say they are correlated at all.

Two tests will be found of general use—the χ^2 test and Fisher's Exact Method for 2 × 2 tables. These tests are well known and simple to calculate and can be found in most elementary text-books of statistics. The best account of them for the non-mathematician is that of Race & Sanger (1954, pp. 59, 126, 272). The χ^2 test is useful for tables with large figures, or with more than four values (such as 3 × 3 tables) provided no entry is less than 5. Fisher's method is useful where one entry is zero or a small number, and is easily calculated using a table of the logarithms of factorials. These tests are not readily misapplied, and are unlikely to give false results.

Having made a significant finding, one has to consider its cause. Statistics will not tell one this. The reason for the correlation may be that the two tests are really testing the same property—for instance caseinase and peptonization in litmus milk. The lack of one enzyme concerned with glucose metabolism might influence apparently unrelated things like the ability to utilize fructose and the ability to grow anaerobically. If two features are always associated throughout bacteria, this may be because they are two properties of the same thing. A similar argument applies to negative features; for instance, since no bacterium is known to require chloroform as a growth factor it would be superfluous to count chloroform-independence as a feature. The safeguard is to study enough features, which will make the uncertainty less important. For reasons of this sort, one should be reluctant to create a division on only two correlated features. Four or five such features (not all belonging to one biochemical process) would seem to be the minimum for safe division.

One more problem will arise, and that is how to express the probability that five or six tests, not just two, are correlated. This can be done if χ^2 values are calculated, since it is permissible to add together the various values for χ^2 and the various values for the degrees of freedom used in this test and then get the probability for $\Sigma\chi^2$ and Σ d.f. which expresses the overall probability. If need be, the χ^2 values can be obtained by special methods with entries below 5.

One of the pioneers in the application of statistics to bacterial classification was Levine (1918), while Dible (1921) applied his methods in a classic paper. It is possible to express, as these authors did, the correlation in terms of coefficients of association, but this leads into a tangle of statistics, since the significance of the figures thus obtained may be doubtful. Such coefficients are usually unnecessary. The instances considered here are tests which score as positive or negative. Tests which give quantitative results can be analysed by the well-known Coefficients of Correlation. For such tests, a scatter diagram is very useful.

Justification. A justification of introducing mathematics into bacteriological classification may be needed. Mathematics and statistics are already firmly entrenched in things like viable counting, but they may seem foreign to the taxonomist. However, in another field, that of blood groups (which at first must have seemed a very non-mathematical subject) the use of methods as simple as those suggested here has caused an enormous expansion of valuable conclusions and of profitable experimentation.

CONCLUSIONS

The development of 'natural' systems of classification is well summarized by Bather (1927), Gilmour (1940) and Turrill (1942). The address of Bather is very valuable and he traces the subject from its inception by Linnaeus (1743; perhaps foreshadowed by Caspar Bauhin), through the systems of Buffon, de Candolle, de Jussieu, Cuvier and Whewell. However, these systems weighted the features according to their relative importance and this produced a source of uncertainty. Importance was never satisfactorily defined, but it clearly cannot be defined as importance in classification, for this is what we wish to discover.

The first taxonomist who conceived of the use of every feature impartially and with equal weight was Michel Adanson (1763). His views were far ahead of his time. He attacked the arbitrary methods of the day and insisted that every part of a plant should be used in making a classification (Préf. p. clv). He constructed in turn all the classifications which he could, each one based on a single part of the plants, and on comparing them he found that some of the classifications divided up the plants in a manner which was almost identical with that of other classifications. These he chose as indicating the most natural groupings, which were therefore groupings based on correlated features of virtually equal weight. He also realized the nature of the dividing lines which were thus made (Préf. p. clxiv). Adanson's views were not highly esteemed, since his contemporaries considered that some features were more important than others, although they could never agree which features these should be. Adanson seems to me to have been right, for the concept of 'importance' seems to play little part in the concept of 'greatest content of information.' The viewpoint of Gilmour (1940) is close to that of Adanson. Gilmour, on epistemological grounds, reaches the same conclusion as is reached here, that the ideal classification is the one with the greatest predictive value. Such a concept is based on giving every feature equal weight. Such classifications may conveniently be called 'Adansonian'. The basic difficulties which remain are to define a 'unit character' and to handle quantitative data, and an attempt has been made to do this in the succeeding paper. In quantitative tests the phrase 'number of features' is not directly applicable, and it would seem necessary to take as units of measurement units of such size that they are in reasonable relation to the range of variation found in the material. We have, in fact, assumed the truth of this thesis by usually adjusting our tests so that the division lies near the centre of variability of the quantitative feature concerned. The use of 'number of features' may also be justified on the grounds that the quantitative differences depend ultimately on qualitative differences in genes or in nucleotides, to which the concept of number is applicable.

Similarity is better defined in terms of the number of features which are not known to be different properties of the same thing. This is to avoid a logical dilemma, e.g. if a single gene mutation causes many diverse effects shall we score this as one difference or as many correlated differences, and the mutant forms as one species or as two? Since the *raison d'être* of a scientific classification is its high content of information (its predictive value) the two forms will be classified as one species, for this procedure gives a greater content of information—viz. it adds that one form can turn into the other (in a sense also they are similar in their potential features). The paradox is that the value of a correlation in classification is lost when our knowledge is sufficient to explain why this correlation exists, but it does not matter that what used to be counted as several correlated features is now counted as a single feature since the function of scientific classification is to enable us to make predictions about unknown and unexplained properties. Similar considerations make one classify insect larvae with the adult forms. However, this point may seem only of academic interest in bacteria.

The most startling new concept in classification is the 'dynamic system' of Hayata (1921). This likens the relationships of plants to a net or a sponge, and springs from the observation that there are many cross-relationships between very diverse families. It is an expression of the fact that similarity is multidimensional; there is one dimension for each feature and to represent it one requires multidimensional space, so that the net or sponge is an oversimplification. Hayata fully recognized that in practice the relationships must be summarized into a hierarchical system, but suggested that instead of a linear array of families it is better to flank each family name with the names (in small print) of the families which are most closely related, i.e. the nearby knots of the net. Hayata's system is well discussed by Du Rietz (1930) and Airy Shaw (1950) who believed that it may have a large part to play in botany. It would also seem particularly appropriate to bacteria and will repay closer study. It has been further developed (Hayata, 1931) to bring it more into line with orthodox genetics.

No mention has been made of evolution, which some believe to be essential to a scientific classification. But, as the Irishman said, there is a red herring in the air and it must be nipped in the bud. One can make a classification without considering phylogeny (indeed the outlines of the classification of higher organisms were laid down before Darwin—see Bather, 1927; Airy Shaw, 1950; Pirie, 1955), although it will necessarily be incomplete. The phylogenist may amplify it and modify it in details. His ability to do this is limited: he cannot deny that the degree of similarity is a good measure of ancestral relationship for he must use this very criterion to choose the members of an ancestral tree. The bacteriologist has no choice in the matter; those who hanker after phylogenetic schemes in bacteria should read the critical review of Sporne (1956) on this problem in angiosperms, in which the fossil record is scanty. He rejects as invalid most of the criteria for primitive features which have been proposed and concludes that a fossil record is needed for the application of the others. The bacterial fossil record is summarized by Pia (1928); some substitute may be provided by bacterial genetics —what may be called 'micro-evolution'.

We have concluded that the *number* of features in common is the basis for deducing similarity but that *correlated* features should be the basis of division into taxa. Does this mean that an indivisible group should always be of lower taxonomic rank than an unrelated but divisible group? I do not think that it should. One species may be divisible into distinct subspecies and races, while another may not and the causes may be various (lack of genetic exchange, restriction to certain microenvironments, etc.). Since the concept of species is an artificial one, there is no need to make divisibility a bed of Procrustes. At present, we have almost nothing to guide us on the rank which should be given to a taxon of bacteria—it is a matter of personal preference. The theoretical method is to lay down that strains of one species should have a certain percentage of common features, but until more work is done upon this it is desirable to observe two working rules. The first is that a mutant should not be given specific rank. Bacteriologists do not do this, but they are sometimes tempted to give specific rank to strains which might be naturally occurring mutants differing from the common type in only a single feature. The second is that one should be chary of separating into different genera bacteria which undergo genetic exchange.

Cowan (1955) posed some interesting questions which it is fashionable to attempt to answer. The last three were admirably answered by Howie in the discussion on Cowan's paper. The first two questions are: (1) Does the hierarchical structure of a Linnaean system satisfy the requirements of microbiologists? (2) Can we accept the species concept, and all that this implies, or must we view our organisms as a huge spectrum composed of gradually merging forms?

The two questions are inter-related, for if bacteria form a continuous spectrum then, since we cannot think in terms of such spectra, we are forced to use a hierarchical system of some kind: we may as well use the Linnaean system as any other, and we may as well use the term species where we can. In fact I do not believe that such a spectrum is so continuous that we can never summarize it.

The spectrum can, however, be summarized in different ways, as Cowan (1956*b*) pointed out, to suit the needs of various workers. van Niel (1946) also suggested a similar approach, but wisely did not call it taxonomy. These alternatives to the ideal classification are often not scientific classifications but are catalogues; in so far as each alternative approaches the ideal classification they will approach one another. They may be necessary steps to that ideal, but they are obsolescent, and should not be indiscriminately called taxonomies, but schemes of division, or keys for identification. A classification based on the number of similar features and their correlation, in other words the classification which would emerge if one fed one's data into a calculating machine, would in theory be the best classification which could be made with the available data. One can instruct the machine to compare each strain with every other and tabulate the number of differences, and then place together into groups those strains with no differences, add to these groups the strains with one difference and so on. Similarity can then be given a numerical value and this allows taxonomic rank to be defined in terms of similarity, as is only logical. An attempt has been made to do this in the next paper (Sneath, 1957). In this sense classification is a branch of mathematics.

The practical man may complain that he does not want a classification based on correlated features (all qualified by the word usually); he just wants a test to identify a pathogenic bacterium. But, if there is no correlation between pathogenicity and any other feature, it simply means that no such test is known. No re-arrangement of the taxonomy will help him, and if he groups all pathogenic strains into one species, this species means no more than 'pathogenic strains of genus A'. Is it heretical to suggest that it is simpler to say this rather than to use a Latin name?

Classifications based on the ideas discussed here might with profit be tried out on viruses and bacteriophages before the taxonomy of these becomes overloaded with preconceived notions of how to do it. Antigenic analysis will be largely used and this will cause some difficulties; but the only logical way would be to assume only such number of distinct antigens as are essential to explain the serological results, that is to use Occam's Razor, and to use these results as the basis for counting similarities and differences.

Will divisions based on correlated features stand the test of time? For the answer we may consider a well-known example such as mammals and birds. New techniques in embryology, biochemistry, cytology and so on reinforce the separation of Aves from Mammalia and the separation becomes more reliable and the classification of the vertebrates becomes more closely knit, precisely because the new discoveries correlate with the old knowledge. Similarly, in the genus *Bacillus* the work of Knight & Proom (1950) on nutritional requirements has reinforced the taxonomy of Smith *et al.* (1946). A bad classification is pulled apart by new discoveries.

Any interest which this may possess is due to stimulating discussions—and arguments—with Drs J. Mandelstam, S. T. Cowan, M. R. Pollock and B. A. D. Stocker and many others, whose help is gratefully acknowledged.

REFERENCES

Adanson, M. (1763). *Familles des plantes*, vol. 1, Préface pp. cliv. *et seq.*, p. clxiii, p. clxiv. Paris: Vincent.

Ainsworth, G. C. (1955). The principles of microbial classification. Host-parasite relationships. *J. gen. Microbiol.* **12**, 352.

An Unofficial Interpretation of the International Rules of Zoological Nomenclature by W. I. Follett, issued by the Society of Systematic Zoology in the United States of America, 1955, 'not published'. 100 pp. (see Anonymous (1956) Nomenclatural Notes: *Int. Bull. bact. Nomen. Taxon.* **6**, 43).

Bather, F. A. (1927). Biological classification: past and future. *Quart. J. geol. Soc. Lond.* **83**, Proc. lxii.

Clarke, P. H. (1955). The principles of microbial classification. Methods for determining the biochemical activities of microorganisms as applied to classification. *J. gen. Microbiol.* **12**, 337.

Cowan, S. T. (1955). The principles of microbial classification. Introduction: The philosophy of classification. *J. gen. Microbiol.* **12**, 314.

Cowan, S. T. (1956*a*). 'Ordnung in das Chaos' Migula. *Canad. J. Microbiol.* **2**, 212.

Cowan, S. T. (1956*b*). Taxonomic rank of Enterobacteriaceae 'groups'. *J. gen. Microbiol.* **15**, 345.

Dible, J. H. (1921). The enterococcus and the faecal streptococci: their properties and relations. *J. Path. Bact.* **24**, 3.

Discussion Meeting of the Society for General Microbiology (1954). The principles of microbial classification. *J. gen. Microbiol.* **12**, 314.

Du Rietz, G. E. (1930). The fundamental units of biological taxonomy. *Svensk bot. Tidskr.* **24**, 333.

Gilmour, J. S. L. (1940). Taxonomy and philosophy, in *The New Systematics*, ed. by J. Huxley, pp. 461–742. Oxford: Clarendon Press.

Hayata, B. (1921). *Icones Plantarum Formosanarum*, vol. 10. pp. 75–95; 97–234. Taihoku: Government of Formosa.

Hayata, B. (1931). Le système dynamique des plantes fondé sur la théorie de la participation. *C.R. Acad. Sci., Paris*, **192**, 1286.

International Bacteriological Code of Nomenclature (1948). Edited by R. E. Buchanan, R. St John-Brooks & R. S. Breed. *J. Bact.* **55**, 287; *J. gen. Microbiol.* (1949), **3**, 444.

Kauffmann, F., Edwards, P. R. & Ewing, W. H. (1956). The principles of group differentiation within the Enterobacteriaceae by biochemical methods. *Int. Bull. bact. Nomen. Taxon.* **6**, 29.

Knight, B. C. J. G. & Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.

Levine, M. (1918). A statistical classification of the colon-cloacae group. *J. Bact.* **3**, 253.

LINNAEUS, C. (1743). *Fragmenta Methodi Naturalis* included at the end of *Genera Plantarum* 2nd ed. (3rd printing). Paris: David.

NIEL, C. B. VAN (1946). The classification and natural relationships of bacteria. *Cold Spr. Harb. Symp. quant. Biol.* **11**, 285.

PIA, J. (1928). Die vorzeitlichen Spaltpilze und ihre Lebensspuren. *Palaeobiologica*, **1**, 457.

PIRIE, N. W. (1955). The principles of microbial classification. Summing-up. *J. gen. Microbiol.* **12**, 382.

RACE, R. R. & SANGER, R. (1954). *Blood Groups in Man*, 2nd ed. pp. 59, 126, 272. Oxford: Blackwell.

ROGERS, L. A., CLARK, W. M. & DAVIS, B. J. (1914). The colon group of bacteria. *J. infect. Dis.* **14**, 411.

SHAW, H. K. AIRY (1950). Post-Darwinian development of taxonomy (botany), in *Lectures on the Development of Taxonomy Delivered in the Rooms of the Linnean Society during the Session* 1948–49, pp. 60–79. London: Linnean Society Pamphlet.

SKERMAN, V. B. D. (1949). A mechanical key for the generic identification of bacteria. *Bact. Rev.* **13**, 175.

SMITH, N. E., GORDON, R. E. & CLARK, F. E. (1946). Aerobic mesophilic sporeforming bacteria. *Misc. Publ. U.S. Dept. Agric.* no. 559.

SMITH, N. R., GORDON, R. E. & CLARK, F. E. (1952). Aerobic sporeforming bacteria *Agric. Monogr.* no. 16, pp. 5–8.

SNEATH, P. H. A. (1957). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

SPORNE, K. R. (1956). The phylogenetic classification of the angiosperms. *Biol. Rev.* **31**, 1.

TOPLEY, W. W. C. & WILSON, G. S. (1929). *The Principles of Bacteriology and Immunity*, 1st ed. p. 202. London: Arnold.

TURRILL, W. B. (1942). Taxonomy and phylogeny. *Bot. Rev.* **8**, 247, 473, 655.

TURRILL, W. B. (1952). Some taxonomic aims, methods, and principles: their possible applications to the Algae. *Nature, Lond.*, **169**, 388.

WHITE, P. B. (1937). Remarks on bacterial taxonomy. *Zbl. Bakt.* (2 Abt.) **96**, 145.

WINSLOW, C.-E. A. & ROGERS, A. F. (1906). A statistical study of generic characters in the Coccaceae. *J. infect. Dis.* **3**, 485.

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SNEATH, P. H. A. (1957). *J. gen. Microbiol.* 17, 201–226

The Application of Computers to Taxonomy

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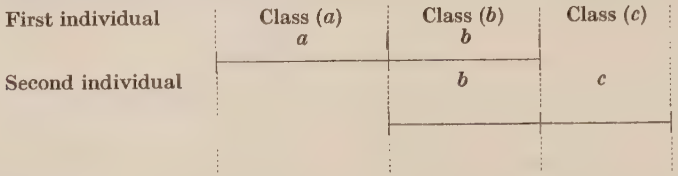
SUMMARY: A method is described for handling large quantities of taxonomic data by an electronic computer so as to yield the outline of a classification based on equally weighted features. This enables *Similarity* to be expressed numerically, and would allow taxonomic rank to be measured in terms of it. An example in bacteria is given, and the results compared with the conventional classification. The method is to count the number of similar and of dissimilar features between strains and to sort the strains into groups whose members have a high percentage of similarities.

The classification of bacteria has reached a point at which there is a need for some mechanical aid to sorting the data. This is because it is difficult to remember the reactions of all the strains in all the tests and therefore one cannot easily separate the strains into valid taxonomic groups. In a discussion on bacterial taxonomy (Sneath, 1957*b*) four main conclusions were reached. First, that the ideal classification is the one which has the greatest content of information. Secondly, that overall similarity was the basic concept of such ideal classifications and that it is measured in terms of the number of similar features possessed by two organisms. Thirdly, that every feature should have equal weight. Fourthly, that division into taxonomic groups is made upon correlated features. Classifications based on the last three principles (which may for brevity be called Adansonian classifications) will contain the greatest content of information.

A development of these principles is that overall similarity may be estimated numerically, and in this paper an attempt is made to do this by using an electronic computer, and to suggest how overall similarity might be used as a criterion of taxonomic rank. The method has been briefly described in a preliminary communication (Sneath, 1957*a*). The first step is to convert one's data into a table of features which are scored as present or absent; for this the term 'feature' must be defined and a method of scoring devised. Secondly the table must be analysed so as to give a numerical value for overall similarity. For each of these steps there are a number of alternatives which are equally valid logically, but in general less convenient than the methods used in this paper.

In order to visualize the position the following statistical model may be made. If one considers an individual as possessing a very large number of features (all of which are observable with the techniques at present available) one could divide the features of two such individuals into three classes: (*a*) those features possessed by the first but not by the second individual, (*b*) those possessed by both individuals, and (*c*) those possessed by the second but not by the first individual. The numbers in the classes could be counted, and shown diagrammatically as below where the numbers of features of the three classes is indicated by the distances, a, b, and c respectively. In this model every feature has equal weight.



Overall similarity is indicated by the degree to which the two solid lines overlap. It may be represented by the fraction $b/(a+b+c)$, and could theoretically vary from 1·0 where the individuals are identical in all features to practically 0 where very different kinds of living creatures are compared. This ratio may be called *Similarity*, or S_t, which is the measure of overall similarity which is adopted in this paper. It is of course clear that the exact value of S_t will depend on the way in which the term 'feature' is defined, but if the same definition is employed consistently, the values will be comparable. S_t therefore can be looked upon as the best measure of overall similarity which could be obtained with present-day techniques.

Since in practice one cannot do all the possible tests, one must obtain an estimate, S, of S_t by taking a sample of observed features and sorting them into classes (a), (b) and (c). Two questions here arise: (1) can one assume that the larger the sample the better this estimate will be, and (2) can one assume that the S values will be fairly stable despite the discovery of new features by techniques which will only be devised in the future? The validity of these assumptions is discussed later, but one may deduce from past experience that the answer to both questions is yes. If it were not so one would find that new discoveries overturned the old classifications, and this does not, I think, generally happen with Adansonian classifications—in fact it is one of the points in favour of them. The relation of one S value to another would be more stable than the S values themselves, as is mentioned in the discussion. Although we cannot obtain absolute and stable values of S, but only approximations, this only amounts to saying that a classification must be made with the data which are available, which is true for any method, whether Adansonian or not.

In the method of analysis used here the only features employed are positive features. No account is taken of negative features, i.e. features which are absent from all the organisms under study. This is desirable since the class of absent features is almost infinite, and one cannot make a logical selection of them. Although this does mean that some negative data are not analysed, it does not reduce the information in the classification. For instance, if one states that strains of *Escherichia coli* are indole-positive, and that strains of typhoid bacilli differ from them in the indole reaction, then it is implied that typhoid bacilli are indole-negative.

This paper is concerned with S. The other observable ratio, of class (a) to class (c) is of less interest, but it might give an estimate of the relative size of the genome (or more strictly of the sum of possessed features or 'phenome'), as measured by the ratio $(a+b)/(b+c)$.

Symbols

Individuals, or in bacteria individual strains, are indicated by numbers. Groups of individuals or groups of strains are indicated by letters. Subscripts are used to indicate the individuals or the groups which are referred to. A single subscript indicates a value within a group; two subscripts separated by a full stop indicate a value which compares two individuals or two groups.

i = the number of individuals, or in bacteria the number of strains.

m = the number of positive features under analysis which are possessed by at least one individual or strain.

For a particular pair of individuals the following symbols are used:

n_s = the number of positive features possessed by both individuals. It does not include the number of features which are not possessed by either individual.

n_d = the number of features possessed by the first individual but not by the second plus the number possessed by the second but not by the first.

$n_f = n_s + n_d$.

S = Similarity, and is n_s/n_f.

D = Dissimilarity, and is n_d/n_f, or $1-S$.

The symbols S and D may be given as decimal fractions or as percentages. The pair of strains referred to is indicated by a double subscript, e.g. $S_{1.2}$. S and D also stand for Mean Similarity and Mean Dissimilarity when they refer to groups of individuals. If the mean is that within a group there are two ways of obtaining it, as is illustrated by Table 1. The first method gives the 'Square Mean' and is obtained by averaging all the values in an $i \times i$ table such as Table 1, including the values for an individual compared with itself. The Square Mean S_a from Table 1 is

$$[(4 \times 1{\cdot}0) + (2 \times 0{\cdot}9) + (2 \times 0{\cdot}8) + (8 \times 0{\cdot}5)]/16 = 0{\cdot}712.$$

The second method gives the 'Triangle Mean' and excludes self-comparisons and also the redundant repetitions (e.g. if $S_{1.2}$ is counted then $S_{2.1}$ is excluded since it is the same thing) and is the average of the S values in the outlined triangle. In this example, Triangle Mean S_a is

$$[(0{\cdot}9 + 0{\cdot}8) + (4 \times 0{\cdot}5)]/6 = 0{\cdot}617.$$

When the mean is that between strains or between groups there is only one way of averaging the values. In this example $S_{1.2}$ is 0·9, while $S_{b.c}$ is $(4 \times 0{\cdot}5)/4 = 0{\cdot}5$. The 'Square Mean' is the more useful as it is additive in certain circumstances, and is distinguished here by the prefix capital *gamma*, e.g. ΓS. The 'Triangle Mean' here has the prefix capital *delta*, e.g. ΔS. ΔS is lower than ΓS but approaches it when i becomes large. ΔS is related to ΓS by the formulae

$$\Delta S = \frac{\Gamma S i - 1}{(i-1)},$$

$$\Gamma S = \frac{\Delta S(i-1)+1}{i},$$

ΔD and ΓD show complementary properties, which may be derived from the relation $D = 1 - S$.

Table 1

S values for strains 1—4, which together form group a. The group is divisible into subgroup b which consists of strains 1 and 2, and subgroup c consisting of strains 3 and 4.

		Strains			
		1	2	3	4
	1	1·0	0·9	0·5	0·5
Strains	2	0·9	1·0	0·5	0·5
	3	0·5	0·5	1·0	0·8
	4	0·5	0·5	0·8	1·0

Definition of the term 'feature'

If one accepts the thesis that the ideal scientific classification is the one which contains in its divisions the greatest amount of information, then one may logically define the word feature in terms of the number of statements which can be made about an organism. The working definition adopted here is that one feature is a thing about which one statement can be made, but that not all features can be counted when creating a classification.

To be counted the statement must be based on observed data and not merely assumed, otherwise it leads to circular reasoning of the kind 'I assume this creature has a mammalian heart and therefore the nature of its heart supports my contention that it is a mammal'.

To be counted the statement must add new information about the organism. Therefore it must not be: (1) A tautology. (2) True by definition, e.g. 'penicillin is the substrate of penicillinase.' (3) True because it describes a property which is known to be obligatory, e.g. 'haemoglobin is red'. (4) A redundant statement which is implied by statements listed previously, e.g. 'growth occurs at 37°' is redundant if one has listed 'growth occurs from 12° to 44°'. (5) A combination of these.

Only positive features can be compared. A positive feature is defined as one which we must assume to be present in order to explain an observation. A negative feature is one for which we have no evidence, i.e. the observations may be explained by the absence of a feature. In bacteriology a negative feature will generally be one which gives the same result as a control test omitting the bacterium; indole-negative, for instance, is a negative feature since it may mean that the bacterium possesses no genes or enzymes which are in any way concerned with indole formation. There are few features which cannot be logically sorted into these two categories. The most difficult are those describing the toxicity of drugs, etc., in which several logical alternatives are possible: these are discussed in the appendix. If two organisms both possess the same positive feature this counts as one similarity; if one possesses it and the other does not this counts as one difference; if neither possess it this does not count at all. This is logical, since it is not pertinent to count 'lack of feathers' as a feature when comparing bacteria with one another, but it would be pertinent in comparing a bacterium with a bird. If one once admits comparisons between negative features one does not know where to stop.

Reduction of bias

Although the observed features of an organism cannot be proved to be unbiased with respect to S_t, one should not introduce deliberate additional bias by using a test which was selected because it readily separates the group of organisms under study. Neglect of this could (in theory) lead to ridiculous results. For example, if one were obsessed with the notion that the typhoid bacillus was in no way related to the colon bacillus one could spend one's life looking for new tests which separated them, and one might obtain a few such tests by trying out a large number of new tests. An analysis with the selected tests would show no similarity at all between the two bacteria. Clearly, one should either exclude all the tests or include all of them. However, there is no reason for assuming that the selected tests would necessarily be biased when studying a distant family such as the micrococci. Usually there is no record in the literature of the tests which were tried out to discover a test of this kind. One should therefore exclude a test which was devised in order to divide the group under study, or whose sensitivity was deliberately adjusted to this end, but may employ it when studying other bacteria. The same objection applies to tests which were devised in order to unify the group under study: e.g. the urease test *adjusted so as to be positive only with Proteus strains is* inadmissible when studying Proteus. The number of such tests is not great, and their inclusion might not in practice matter very greatly. The number of tests used must be adequate. The accuracy of S is roughly indicated, though in general underestimated, by its standard deviation $\sqrt{[(S \times D)/n_f]}$. Ideally one should employ all the unbiased tests one can, but if one must make a selection of them, it seems wisest to pick a random selection, taken from many different classes such as morphology, carbohydrate fermentation, etc.

The programme of analysis

A simple example of the way in which the value for S is obtained is shown below. Three bacteria have the following 'IMVIC' reactions:

	Indole	Methyl Red	Voges-Proskauer	Citrate
Strain 1	+	+	–	–
Strain 2	+	+	–	–
Strain 3	–	+	–	+

On comparing a pair of strains one first lists the number of tests in which both are positive. Thus strains 1 and 2 are both positive in two tests, while strains 1 and 3 are both positive in the Methyl Red test only. These comparisons are made for all possible ways of pairing the strains, and the resulting table of n_s values is as follows. Note that where both strains are negative, this is not included in finding n_s.

	Strain 1	*Strain* 2	*Strain* 3
Strain 1	2	2	1
Strain 2	2	2	1
Strain 3	1	1	2

One then compares the strains and lists the number of tests in which one strain is positive and the other strain is negative. Thus, strains 1 and 3 show two differences (the indole and the citrate tests). These figures give the table of n_d values shown below:

	Strain 1	*Strain* 2	*Strain* 3
Strain 1	0	0	2
Strain 2	0	0	2
Strain 3	2	2	0

The value of $n_s/(n_s+n_d)$ can then be obtained for each way of pairing the strains, giving the table of S values.

	Strain 1	*Strain* 2	*Strain* 3
Strain 1	2/2	2/2	1/3
Strain 2	2/2	2/2	1/3
Strain 3	1/3	1/3	2/2

The S values are conveniently expressed as percentages:

	Strain 1	*Strain* 2	*Strain* 3
Strain 1	100	100	33·3
Strain 2	100	100	33·3
Strain 3	33·3	33·3	100

Part of the tables of n_s, n_d and S is redundant, and may be omitted, since it is obvious that any strain when compared with itself will have $S = 100\,\%$ while the upper right-hand part of the table is a 'mirror image' of the lower left-hand part. Of course no useful results can be expected from considering only four tests, but the same method is used for larger numbers of features.

Preparation of data. The data are tabulated with features in the columns and individual strains in the rows (an $i \times m$ table). Strains are scored as + indicating presence of the feature, or as − indicating its absence. Where no datum is available the space is marked NC (standing for 'No comparison').

Sorting of quantitative data. It is difficult to score quantitative data in an entirely logical fashion, and an empirical method has to be used. The basic principle is to assume the existence of only such features as are necessary to explain the experimental findings. The quantitative results are first inspected to see how many steps can be clearly distinguished in the material under study by the experimental method, so that one can be confident that there is a real difference between strains in one category and strains in another category, after allowing for biological and experimental error. Strains which are intermediate between two categories, which will occur if there is a continuous curve of variation, must perforce be allocated arbitrarily, but one trusts to the large number of features under analysis to prevent systematic errors from this cause. One must now find the numbers of similarities and differences which are implied by the categories. To do this, it is first necessary to make one of two assumptions, either that features are additive in their properties (which would seem to be a basic assumption in using correlation coefficients), or that they are not. Unless one can decide between these alternatives from the data, which will be very rarely possible, this choice is necessarily an arbitrary one. I feel that it is best to assume that features are not additive, and this leads to the following method of scoring.

Each step is assumed to be under the control of a feature which determines the quantity of the characteristic, and which is different from the features determining the quantity of the other steps. However, all those strains which give a detectable reaction at all resemble one another in this respect. Each such strain must therefore possess a minimum of two features (or two subfeatures, which by the working definition of feature, may be regarded as distinct features)—one allowing expression of the characteristic, and one determining the quantity. An example is as follows. Five strains produce respectively no detectable indole, 5, 10, 10·5 and 20 μg./ml. of indole under defined conditions. The difference between 10 and 10·5 μg./ml. is considered to be of no significance because of the errors of the test. The first strain is allotted no features. Strains 2–5 are allotted a common feature, A, for indole-production, and are allotted features B, C and D, for the amount of indole which is produced. The constitution of the strains is provisionally written as follows:

Strain 1		(producing no indole)
Strain 2	AB	(producing 5 μg./ml.)
Strain 3	AC	(producing 10–10·5 μg./ml.)
Strain 4	AC	(producing 10–10·5 μg./ml.)
Strain 5	AD	(producing 20 μg./ml.)

One has, however, no reason for assuming the existence of feature B, since one could not distinguish, in this instance, between a strain which was AB and a strain which was A. One therefore deletes feature B. We have also assumed that the effect of features is not additive, and it is therefore impossible to distinguish between AD and ACD as the constitution for strain 5: that is, the feature C cannot be scored either as positive or negative in strain 5, and must be scored NC. Also, strain 1 does not, by definition, possess feature A, and therefore it is meaningless to ask whether it has features C or D, so that these two features must be scored NC. The strains are then coded as follows:

	Features		
Strains	A	C	D
1	−	NC	NC
2	+	−	−
3	+	+	−
4	+	+	−
5	+	NC	+

Although C is not detectable in strain 5, yet one can say that D is absent from strains 2–4, because of the way we have defined the features. Similarly, both C and D are absent from strain 2.

This scheme (which is referred to in the Appendix as method C) fulfils two necessary conditions. First, that the S values between a pair of strains are the same however many steps are distinguished: this can readily be seen by extending the steps (so that a sixth strain, for instance, producing 50 μg./ml. indole, this quantity being controlled by a new feature E, would score +, NC, NC, + against the features A, C, D, and E respectively). Secondly, that when the test is turned into a purely qualitative test, the strains will score in the same manner as they would have if the test had never been made a quantitative test. For instance, in the above example, if the division between indole negative and indole-positive lies at 7·5 μg./ml. when using a qualitative indole reagent, then the strains score as follows:

	Indole		Indole
Strain 1	–	Strain 4	+
Strain 2	–	Strain 5	+
Strain 3	+		

This is because we are now only able to observe the presence of AC or of AD, and we have therefore no warrant for assuming the separate existence of feature A, and we cannot distinguish between features C and D: the feature 'indole' thus means 'C or D'. This, the simplest way of scoring a test, is referred to in the Appendix as method A.

There are a few tests in which one should retain the feature called B in the above example. This is where there cannot be a strain which is A negative—for instance in coding the length of a bacterium. This is referred to in the Appendix as method B.

Conversion of data. The data in the large $i \times m$ table are now punched on to teleprinter tape or cards in the manner required for the computer which is used.

Method of analysis. The method given below is converted into appropriate instructions for the computer, and then forms the 'computer programme', which is fed in with the data. The steps are:

(1) The $i \times m$ table is stored.

(2) Each pair of strains in all possible combinations is compared in turn, and the number (n_s) of similar features in which both strains of the pair score +, and the number (n_d) of different features in which one strain scores + and the other – are counted. The machine makes no comparison of a – with a –, nor of either a + or a – with a space marked NC. The value of S is calculated (being $n_s/(n_s+n_d)$) for each pair.

(3) The S values are tabulated in an $i \times i$ table, and this table may be printed for checking. It need not be the full table, but can be the 'non-coincidental triangle' referred to under Triangle Mean in the section on symbols.

(4) The $i \times m$ table is forgotten, and only the table of S values is used.

(5) The machine sorts the strains into groups, working in small steps of S. Any pair of strains with S values of over 0·99 are placed together, and these groups (each consisting of almost identical strains) are printed.

(6) The machine repeats step 5, working at S values of over 0·98, and adds any strains (which have an S value to *any* strain already grouped and printed of more than 0·98) to the earlier groups, and prints them. It also creates and prints any new groups it finds.

(7) The machine repeats step 6 for S values of 0·97, 0·96 and so on until all strains have been grouped and printed, when it is instructed to stop.

(8) The results indicate the groups which are present and roughly their relationships. One might for instance obtain a record:

S	Strains	
0·99	1, 2	
0·98	1, 2, 3	4, 5
0·97	1, 2, 3	4, 5
0·80	1, 2, 3, 4, 5	

This record shows that strains 1, 2 and 3 form a group (*a*), and that strains 4 and 5 form another group (*b*). The two groups are not very similar, since they do not fuse into one until the S value falls to 0·8.

(9) The machine may now be asked to calculate intragroup and intergroup Mean Similarity values and to print the S value table in a rearranged form. The Mean S values give a truer picture than those indicated in step 8, since the machine is sorting strains by the smallest differences between strains and not by Mean values. The example above might yield the better values

$$\text{Mean } S_a = 0{\cdot}988$$
$$\text{Mean } S_b = 0{\cdot}981$$
$$\text{Mean } S_{a.b} = 0{\cdot}781$$

S_a and S_b will be either ΓS or ΔS values according to the instructions.

These results may now be converted into the more familiar and understandable form of a taxonomic tree. We might for instance conclude that Groups (*a*) and (*b*) are sufficiently similar (as indicated by $S_{a.b}$) to belong to one genus, but that they are different species, viz:

Genus *X*

Species *Y* (Group *a*) Species *Z* (Group *b*)

It must be pointed out that it is impossible to summarize an S value table without loss of some information. This is because the S values are quantities in multidimensional space, and the sorting procedure is forming hierarchical groups in a few dimensions. Nevertheless, it is impossible to remember tables of S values, and if anyone else is to understand or to use the classification, these hierarchies must be formed, and one must strike a balance between convenience and accuracy.

This procedure clearly measures overall similarity based on features of equal weight: in addition, the divisions are based on correlated features.

Results of the worked example given in the appendix

The appendix contains the details of the analysis of a study of 45 strains of bacteria. Thirty-eight of these were strains of the genus *Chromobacterium*, which had previously been found to belong to two groups, a mesophilic group (18 strains) and a psychrophilic group (20 strains) (Sneath, 1956*b*). The remaining strains were not of this genus but were included for comparison.

The sorting procedure (steps 5–8 above) correctly sorted the strains into the expected groups as shown below.

Group *a*	18 mesophilic strains of *Chromobacterium*
Group *b*	20 psychrophilic strains of *Chromobacterium*
Group *c*	2 strains of *Serratia marcescens*
Group *d*	2 strains of *Pseudomonas aeruginosa*
Group *e*	1 strain of 'Chromobacterium viscosum'
Group *f*	1 strain of 'Chromobacterium iodinum'
Group *g*	1 strain of 'Chromobacterium ianthinum'

This may be seen from Fig. 1, which is a graphical representation of the sorting procedure. It is seen that at the level of $S = 100\,\%$, no groups are formed (or alternatively, one might say that every strain formed a separate group containing only itself). At $S = 95\,\%$ the two strains of *Serratia* form a group. At $S = 93\,\%$ two of the mesophilic strains, MK and DK, form a group to which other mesophilic strains are added as S decreases. Similarly, at $S = 92\,\%$ the psychrophilic group begins to form. Strain FH is evidently an abberrant strain of group *a*, and strain NT an abberrant strain of group *b*, since they are added at low values of S.

The groups *a* and *b* remain distinct until $S = 64\,\%$, when they fuse together by virtue of the pair SH.HD (which has $S = 64\,\%$), the highest value of S for any comparison of a mesophil with a psychrophil. A little below this, groups *c* and *d* fuse with groups *a* and *b*, as does the group *g* represented by only the single strain TI. The two diphtheroid-like organisms of groups *e* and *f* only fuse with the rest at the level of $S = 44\,\%$: this is expected, since they appear to be distantly related to the other bacteria studied. They are, in addition, not very similar to one another, since for the comparison GR to TE the S value is 65 %. A cleft between two groups in Fig. 1 shows that the groups are completely distinct at S levels above that of the bottom of the cleft, since the bottom of the cleft indicates the highest S value found in comparing strains of one group with strains of the other.

The full table of S values (Step 3 above) was rearranged, so that the strains were in the same order as they are in Fig. 1: this table is shown in the Appendix (Table 7 and diagrammatically in Fig. 4). This full table does not force any major modifications of the conclusions reached from Fig. 1. Only minor points emerge—one might for instance judge that Fig. 1 over-emphasizes the abberrancy of strain FH and under-emphasizes the abberancy of strain AM.

A few subgroups may be noted—thus strains MK, DK, TB, TA, TV, and LG and strains RT, SH, and LW form subgroups of group *a*, and similar sub-

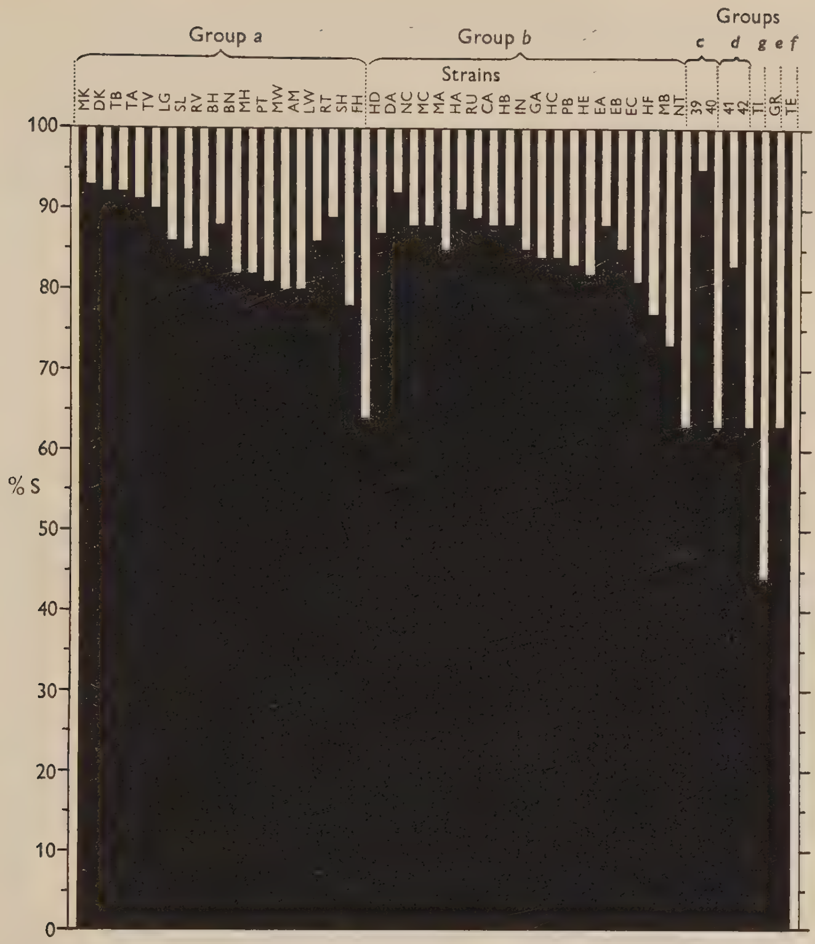


Fig. 1. Diagram of the results of the sorting procedure on the forty-five strains used in the worked example.

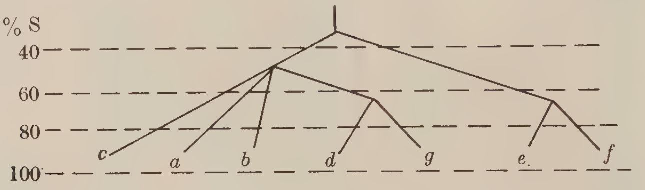
Table 2. *Mean % of S values for the groups a–g*

The S values in the squares are those of the combination implied from the groups tabulated in the row and column. Thus $S_{b.c}$ is 40·1%. The value for a group compared with itself is the ΓS value.

Group	*a*	*b*	*c*	*d*	*e*	*f*	*g*
a	77·2	—	—	—	—	—	—
b	51·5	75·9	—	—	—	—	—
c	54·9	40·1	97·5	—	—	—	—
d	52·3	52·7	54·0	91·5	—	—	—
e	40·0	32·4	32·5	39·0	100	—	—
f	34·3	30·0	29·5	37·0	63·0	100	—
g	52·4	52·5	44·0	61·5	38·0	42·0	100

groups are seen in group *b*. One might consider these to be rather indistinct varieties or subspecies. The suggested neotype strains (Sneath 1956*a*, *b*) MK and HB are typical members of their group.

The sorting procedure is clearly making sense and not nonsense, but there is one important difference between these results and the conventional classification. One sees from the mean *S* values for the groups shown in Table 2 (and it is also apparent in Fig. 1) that the groups *a*, *b*, *c* and *d* appear to be about equally related to one another. This is shown diagrammatically below:



That is to say, the two groups of *Chromobacterium*, groups *a* and *b* which have been considered by most authors including myself (Sneath, 1956*a*, *b*) to constitute two species within one genus, are as distantly related as *Pseudomonas* is to *Serratia* which are usually regarded as belonging to different families (the Pseudomonadaceae and Enterobacteriaceae respectively). Leifson (1956) has already pointed out that the species of *Chromobacterium* are very different, and has suggested that they might be best raised to generic rank. However, one could also argue that the families Pseudomonadaceae and Enterobacteriaceae should be reduced to the rank of genera. These two families may not be as distinct as is commonly assumed, for there are an increasing number of reports of organisms which are intermediate between the typical pseudomonads and the typical Enterobacteriaceae (e.g. Leifson & Hugh, 1953; Crawford, 1954; Asai, Okumura & Tsunoda, 1956; Sreenivasan & Venkataraman, 1956), and the two families are now beginning to look like arbitrary categories based only upon whether the flagella are polar or peritrichous. This feature itself is less simple than was thought, since Leifson (1956) and Sneath (1956*c*) have shown that *Chromobacterium* sp. may have both polar and peritrichate flagella.

It seems to me that bacteriologists are apt to assume that two bacteria which differ in a striking feature such as their pigment are also different in many other uninvestigated features, and that this is the reason why *Chromobacterium*, *Pseudomonas* and *Serratia* are considered to be distantly related. But this assumption is quite unproved. It is possible that a variant of *Pseudomonas* which produces violacein, or a strain of *Chromobacterium* which produces fluorescein, might be discovered, which would then suggest that the two genera were very closely related. Similarly, the tacit assumption that the type of flagellation reflects a fundamental difference between the Enterobacteriaceae and the Pseudomonadaceae (or more accurately that there are many features correlated with flagellation), is unproved, and is now looking unsafe.

The example given here may not be a very good test of the method, since sharply defined groups of bacteria were used, and the least one would expect is that the method should separate them. The example does, however, suggest that the method does not disrupt sound taxa.

DISCUSSION

Probably the most controversial point in the method described here is the dictum that every feature should have equal weight. This is, however, not a matter for experimental proof; it is for taxonomists to decide whether they want the kind of classification which is given by equally weighted features. The viewpoint taken here is that it is impossible in practice to decide on relative weight, and this avoids much sterile argument.

The definition of the term feature is a makeshift. It may be possible in the future to re-define it in terms of genes and perhaps nucleotides; this will not effect the basic concepts of Adansonian methods but may simplify them.

The S values are analogous in some respects to the 'coefficient of racial likeness' which has been developed in anthropology by Karl Pearson and others (see discussion by Rao, 1948). In the field of entomology Sturtevant (1939, 1942) made an analogous approach to the problem of classification, and Dr R. R. Sokal informs me that he has developed a similar method (Sokal, 1956); Sokal & Michener, 1957*a*, *b*).

Stability of S values

Reference was made earlier to the question of whether one obtains a better estimate of S_t as one increases the number of tests under analysis. This is not, as at first sight, a simple question. The tests may be biased because they contain a high or a low proportion of tests in class (*b*) (see p. 201), and this can occur by the accidents of history. For example a school of workers might develop many simple tests for biosynthetic pathways of the amino acids, and in a group of prototrophic bacteria most strains would be positive in every test. This would raise the S values in the entire group. A further development might reduce the S values again (e.g. by studying the antigenic differences of the enzymes concerned). One cannot prove that either estimate was the better, although by definition S will equal S_t when all possible tests are performed. This means in practice that one cannot extrapolate an observed tendency to obtain S_t. Similarly, new technical developments will cause shifts in S values and one cannot extrapolate to obtain S_t values that might be obtained with tests that will be invented in the future. The S values are useful nevertheless because they are the best estimates available at the time.

It would be inconvenient if S values should fluctuate too widely. However we would expect some constancy in the relations between S values from different analyses since it is usual to find that organisms which are similar in a given set of features are also similar in other features. If this were not true, one would observe individuals with all possible combinations of features—birds with fur and such-like chimeras. One does not observe this even in bacteria (though this has been disputed) since we do not find streptococci causing tetanus nor salmonellas causing diphtheria: it is also evident that some of the possible combinations would cause inviability.

Thus, if one had three strains of typhoid bacillus, strains 1–3, and analysed them using a hundred current tests one might obtain the following S values—$S_{1.2}$ 90 %, $S_{2.3}$ 80 %, $S_{1.3}$ 72 %. Now if new tests were devised for a hundred biosynthetic enzymes, and a new analysis was made including these, one might observe that strain 1 had all of these. If this were so then I think that one would find that strains 2 and 3 also possessed all the enzymes, or the great majority of them. The second analysis would then give $S_{1.2}$ 95 %, $S_{2.3}$ 90 % and $S_{1.3}$ 86 %. In this particular instance the *relative* D values are the same, since $D_{1.2}$, $D_{2.3}$ and $D_{1.3}$ are respectively 10, 20 and 28 % on the first analysis and 5, 10 and 14 % on the second analysis. This might be commonly true in practice, but when it was not, one would still be able to calibrate one scale of S values in terms of another with reasonable accuracy provided there were several strains which had been studied in both analyses.

Taxonomic rank

If one bases taxonomy upon the concept of overall similarity it is illogical to base taxonomic rank upon other criteria. The rank of *species* is commonly defined in terms of interbreeding, geographical distribution, divisibility into subspecies, etc., but these criteria are usually not applicable to higher ranks of taxa. Similarity is applicable at all levels of rank. It is true that the use of Similarity to define taxonomic rank would cause some changes. For instance, a group which is presently called a species might become a genus, but this is only a different way of stating the same thing; instead of being a species (i.e. a group showing free interbreeding) which is very variable in its features it would now be called a genus (i.e. a group showing much variability) which shows free interbreeding. In bacteriology our criteria for taxonomic rank are so poor that a great simplification would result from basing rank upon Similarity, as expressed by S. By ignoring comparisons between negative features, the S values for most bacteria will not be altered by the discovery of bacteria with very unusual positive features, nor will they be affected by positive features which are absent throughout bacteria, such as feathers.

In order to use S values as criteria of rank one must select a few type strains as reference points for the ranks, and one can then decide the rank of other strains and taxa. Thus if one takes a strain of *Bacillus anthracis* and a strain of *Bacillus subtilis* and says that they belong to the same genus but to different species one can decide from the S values whether other strains should be regarded as species, subspecies or varieties. These type strains would then be used in subsequent analyses as reference points. This would provide a practical solution to the problem of reaching general agreement on the criteria of taxonomic rank in bacteria. Clearly one of the first steps is to make an analysis using a few strains from each family of bacteria (including wherever possible the type strain of the nomenclatural type.). This would provide a skeleton on which to build other analyses, and would hasten the international agreement of type strains of the better-known bacteria, a reform which is overdue.

Practical applications in bacteriology

The massive data now accumulating in the records of reference laboratories will never be adequately analysed without mechanical aid. These data can be analysed in a 'scientific' manner if the machine is correctly instructed. It is true that the data must be copied into large tables or typed on to computer tape or cards, but this is a necessary preliminary to any form of analysis, and there is no reason why they should not be compiled piecemeal and then joined together in the right order. Once a machine is programmed to the problem it can analyse subsequent examples without delay, and the computer programme can be readily translated into the code required for a different computer. The only limitations are where the problem is basically different, or where the data are too numerous for the machine to handle. If the number of tests is small then the data will not be worth analysis however numerous are the strains, but bacteriologists could build up suitable data by taking a random selection of their strains for a bigger testing routine.

Antigenic analysis would provide a great number of possible features. The data must be codified in terms of antigens whose presence is warranted by the serology, i.e. numbered or lettered antigens. These may however be biased features, since in many instances the antisera have been cross-absorbed so as to make them efficient at revealing antigenic differences within a particular taxon. It is not permissible to include features which are just thought to be likely, e.g. R antigens in Salmonella are not demonstrated in routine work, and one is not entitled to assume their presence in all the strains. This rule is important, since this method of analysis is intended to give the best estimate of overall similarity which is possible from the data available, and therefore guesswork must be excluded. Admittedly, a shrewd taxonomist might make a better estimate by guessing, but much of present-day bacterial taxonomy is an art based on guesswork, conscious or unconscious, and the results have not been encouraging.

One other point requires emphasis. The analysis is intended to *produce* taxonomic groups and not to *identify* the groups. It is therefore not necessary to employ tests which are standardized for use with all bacteria, though they must of course be standardized throughout the group under study. Different programmes could employ very diverse selections of features and still yield useful values of S. The tests most useful for identification may be obtained by inspection of the data and included in the description of the groups which were distinguished by the analysis

The method suggested in this paper is applicable to any group of living creatures. Although the data would often be less easy to codify there is no difference in essentials. In higher organisms one can form, by looking at morphology, an intuitive idea of the overall similarity, which makes their classification much easier than that of bacteria, but one could use the computer to obtain numerical values if one wished. For instance a collection of thrushes, blackbirds and ostriches would be correctly sorted by the computer into these three groups; precision could be given to the vague statements that the first two are very similar birds but ostriches are very different by estimating Mean Similarities, such as for thrushes versus blackbirds $S_{t.b}=0{\cdot}92$, for thrushes versus ostriches $S_{t.o}=0{\cdot}62$ and for blackbirds versus ostriches $S_{b.o}=0{\cdot}63$.

For bacteria and viruses the method could be widely used, but it would mean that workers would have to study a few strains at a time very thoroughly and build up the classification piece by piece. It would be 'classification from below' (White, 1937), and though slow it would, I believe, be sure.

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REFERENCES

Asai, T., Okumura, S. & Tsunoda, T. (1956). On a new genus, *Kluyvera*. *Proc. imp. Acad. Japan*, **32**, 488.

Crawford, I. P. (1954). A new fermentative pseudomonad, *Pseudomonas formicans* N. sp. *J. Bact.* **68**, 734.

Leifson, E. (1956). Morphological and physiological characteristics of the genus *Chromobacterium*. *J. Bact.* **71**, 393.

Leifson, E. & Hugh, R. (1953). Variation in shape and arrangement of bacterial flagella. *J. Bact.* **65**, 263.

Rao, C. R. (1948). The utilization of multiple measurements in problems of biological classification. *J. R. statist. Soc.* B, **10**, 159.

Sneath, P. H. A. (1956*a*). Conservation of the generic name *Chromobacterium* and designation of type species and type strains. *Int. Bull. bact. Nomen. Taxon.* **6**, 65; and *errata* ibid. **6**, 157.

Sneath, P. H. A. (1956*b*). Cultural and biochemical characteristics of the genus *Chromobacterium*. *J. gen. Microbiol.* **15**, 70.

Sneath, P. H. A. (1956*c*). The change from polar to peritrichous flagellation in *Chromobacterium* spp. *J. gen. Microbiol.* **15**, 99.

Sneath, P. H. A. (1957*a*). Prejudice in bacterial classification. *J. gen. Microbiol.* **17**, ii.

Sneath, P. H. A. (1957*b*). Some thoughts on bacterial classification. *J. gen. Microbiol.* **17**, 184.

Sokal, R. R. (1956). Quantification of systematic relationships and of phylogenetic trends. *Proc. IXth Int. Congr. Ent.* (in the Press).

Sokal, R. R. & Michener, C. D. (1957*a*). A quantitative approach to classification. *Evolution* (in the Press).

Sokal, R. R. & Michener, C. D. (1957*b*). A statistical method for evaluating systematic relationships. *Kans. Univ. Sci. Bull.*, (in the Press).

Sreenivasan, A. & Venkataraman, R. (1956). Occurrence of paracolon bacteria in a tropical marine environment and their classification. *Nature, Lond.* **178**, 547.

Sturtevant, A. H. (1939). On the subdivision of the genus *Drosophila*. *Proc. nat. Acad. Sci., Wash.* **25**, 137.

Sturtevant, A. H. (1942). The classification of the genus *Drosophila*, with descriptions of nine new species. *Univ. Tex. Publ.* no. 4213, p. 6.

White, P. B. (1937). Remarks on bacterial taxonomy. *Zbl. Bakt.* (2 *Abt.*), **96**, 145.

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APPENDIX

Details of analysis of the example

The data are from protocols used in a study of the genus *Chromobacterium* and most have been already published, together with details of the techniques (Sneath, 1956*b*). In some tests the reactions of every strain were not given, but they can be obtained from Figs. 2 and 3 and the details given below.

The data were entered in a table of strains versus features (an $i \times m$ table) in the form of entries of +, – or NC. This table in a modified form (see below) is given in Figs. 2 and 3. Features which were negative with all of the 45 strains were omitted, and a few data which could not be easily scored, such as the source of the strains, were also omitted. Conscious bias was avoided as far as possible by the following procedures. (1) Any test which had been devised, or selected by trial, or adjusted in sensitivity, because it readily separated or united some of the taxa under study was excluded. (2) standard techniques were used wherever possible (e.g. methylene-blue reduction). (3) Where observations were made at various times (e.g. gelatin liquefaction) the scoring was based, when possible, on the time required to obtain a definite positive result. (4) Where observations were made at different times or by different techniques but it was not possible to score them as in (3) (e.g. the size of cells after 18 and 96 hr. incubation are dependent on one another to some extent, but one cannot tell to what extent), then one time or one technique was selected at random by coin-spinning, and only these data were used. All tests were performed at 25° unless otherwise stated.

One hundred and five features were used. Quantitative characteristics were arbitrarily divided into a few steps for scoring. The letters after the feature numbers refer to the method of scoring described on p. 208.

Morphological characteristics (features 1–24)

All are from nutrient agar plate cultures after 18 hr. incubation.

Length of organisms. Features 1–5 (B) scored by method B as shown below:

	Feature				
	1	2	3	4	5
Up to 2μ	+	+	–	–	–
2 to $2{\cdot}9\mu$	+	NC	+	–	–
3 to $3{\cdot}9\mu$	+	NC	NC	+	–
Over 4μ	+	NC	NC	NC	+

Breadth of organisms. Features 6–9 (B). Scored in the same way as for length of organisms, with three steps (up to $0{\cdot}7\mu$, $0{\cdot}7$–$0{\cdot}9\mu$, over $0{\cdot}9\mu$).

Rounded ends. Feature 10 (A).

Bulged or clubbed ends. Feature 11 (A).

Chains of organisms readily seen. Feature 12 (A).

Palisade arrangement of organisms. Feature 13 (A).

Marked curvature of rods (if length was less than 2μ this could not be estimated and was scored NC). Feature 14 (A).

Pleomorphic forms prominent. Feature 15 (A).

Presence of polar flagella. Feature 16(A).

Presence of lateral flagella. Feature 17(A).

Tufts of polar flagella (i.e. lophotrichous arrangement). Feature 18(A). If there were no polar flagella this was scored NC.

Prominent barred or bipolar staining. Feature 19(A).

Gram-positive staining. Feature 20(A). (Gram-positive granules were ignored.)

Capsule visible by Hiss's stain. Feature 21(A).

Microscopic fat in the organisms by Burdon's stain. Features 22–23(C). This was scored as below:

	Feature	
	22	23
Fat scanty or none	−	NC
Moderate fat	+	−
Abundant fat	+	+

Metachromatic granules (i.e. blue granules) clearly visible by Neisser's stain. Feature 24(A).

Cultural characteristics (*features* 25–40)

I. Nutrient agar plate cultures.

Gelatinous consistency of colonies after 7 days' growth. Features 25–26(C). Graded into none, slight, and marked.

Opaque colonies after 2 days' growth. Feature 27(A) (transparent or semi-transparent colonies scored as −).

Degree of production of the pigment violacein after 7 days. Features 28–29 (C). The scoring was by method C modified as follows since 'detectable violacein' is scored under Feature 63.

	Features	
	28	29
Moderate violacein	+	−
Abundant violacein	NC	+

II. Colony form in gelatin plate surface cultures after 4 days at 20° (the scoring is arbitrary)

Smooth Surface. Feature 30(A).

Lobate or erose edge. Feature 31 (A).

III. Other media

Rate of liquefaction in gelatin stab cultures at 20°. Features 32–35(C). Divided into five steps—no liquefaction in 14 days, liquefaction within 14 days, within 8 days, within 5 days and within 2 days. The last four steps correspond to features 32, 33, 34, 35 respectively; the first step is scored as negative for feature 32 and NC for the others.

Degree of digestion of Löffler serum slope in 6 days. Features 36–37(C). Divided into slight or none, moderate and marked.

Ability to grow on potato slope. Feature 38(A).

Degree of violet pigment on potato slope after 14 days. Features 39–40(C). Divided into none, slight and marked.

Resistance tests (*features* 41–47)

The coding of these tests is arbitrary, since there are a number of logical alternatives. An organism which is killed by a certain treatment must possess at least one characteristic (and one assumes only one if there is no evidence to the contrary) which is both essential to life and sensitive to the treatment. These two properties may be regarded as sub-features, and called E and S: a sensitive organism is then $E+$ $S+$. It seems most logical and convenient to consider them as positive features. A resistant organism may be assumed to lack one of the subfeatures, so that it is either $E-$ S NC, indicating that the sensitive characteristic is not essential, or E NC $S-$ indicating that the characteristic may be essential but is not sensitive to the treatment. If one uses the first convention, then one can code the strains by method C, in which a number of different degrees of sensitivity are indicated by S_1, S_2, etc., and this seems the simplest method, e.g.

	Features		
	E	S_1	S_2
Resistant strain	–	NC	NC
Moderately sensitive strain	+	+	–
Highly sensitive strain	+	NC	+

Heat resistance. Features 41–43(C). Viable cells in one loopful of a broth culture after heating at 56° for 5 and 15 min. scored as follows:

	Features		
	41	42	43
No viable cells after 5 min.	+	NC	+
Viable cells after 5 min. but not after 15 min.	+	+	–
Viable cells after 15 min.	–	NC	NC

Phenol resistance: viable cells in one loopful of broth culture containing 1% (w/v) of phenol after 5 and 10 min. at 20°. Features 44–46(C) scored as below:

	Features		
	44	45	46
No viable cells after 5 min.	+	NC	+
Viable cells after 5 min. but not after 10 min.	+	+	–
Viable cells after 10 min.	–	NC	NC

Penicillin sensitivity: growth inhibited for 2 days by 100 units/ml. of sodium benzylpenicillin in nutrient agar plates. Feature 47(A).

Metabolism and nutrition (*features* 48–70)

Anaerobic growth: visible growth after 4 days on anaerobic nutrient agar plate. Feature 48(A).

Upper temperature limit for growth: 7 days on moist nutrient agar slopes, except for readings at 44° which were made after 4 days. Features 49–53(B). This test is scored by method B, since the characteristics concerned must all resemble one another in that they are all connected with growth at high temperatures. The scoring was as follows:

	Features				
	49	50	51	52	53
Visible growth at 25°	+	+	−	−	−
Visible growth at 30°	+	NC	+	−	−
Visible growth at 35°	+	NC	NC	+	−
Visible growth at 44°	+	NC	NC	NC	+

Lower temperature limit for growth: 7 days on moist nutrient agar slopes. Features 54–57(B). The scoring is by the same method as the preceding test. The features 54 and 55 to 57 describe respectively the common subfeature concerned with low temperature growth, and subfeatures for growth down to 16°, 10° and 4°.

Rate of citrate utilization: Simmon's medium containing 0·01 % (w/v) yeast extract to supply growth factors. Features 58–59(C). The results were graded into none, slow and fast, according to whether the medium became blue in more than 5 days, within 5 days or within 2 days respectively.

Tolerance of NaCl. Features 60–61(C): growth on NaCl nutrient agar after 7 days. This test may be regarded as a toxicity test. Although one might postulate many features, since some highly halotolerant bacteria are known, the features used are only those implied from the results in this study. The scoring is as follows:

	Features	
	60	61
No growth on 3 % NaCl nutrient agar	−	NC
Growth on 3 % NaCl but not on 6·5 % NaCl agar	+	−
Growth on 6·5 NaCl agar	+	+

Production of detectable HCN in aerobic agar cultures. Feature 62(A). Production of pigments. Features 63–69(A). These features 63–69 describe the production of detectable amounts of the pigments violacein, prodigiosin, pyocyanin, fluorescein, the blue-violet 'Chromobacterium viscosum' pigment, iodinin and the magenta pigment of strain TI respectively under appropriate conditions. Some pigments would mask others (e.g. violacein might mask prodigiosin) and some NC entries are therefore given. In the bacteria studied here there is no point in allotting features for the conditions for pigmentation, so these are omitted.

Growth in serial subculture in Koser's citrate medium. Feature 70(A). A positive reaction in this test presumably implies the possession of a large number of features allowing the biosynthesis of amino acids, nucleotides, etc. but as this was not investigated in this study it must be counted as a single feature.

Biochemical reactions (*features* 71–105)

Ability to ferment glucose anaerobically in Hugh & Leifson medium within 7 days. Feature 71(A).

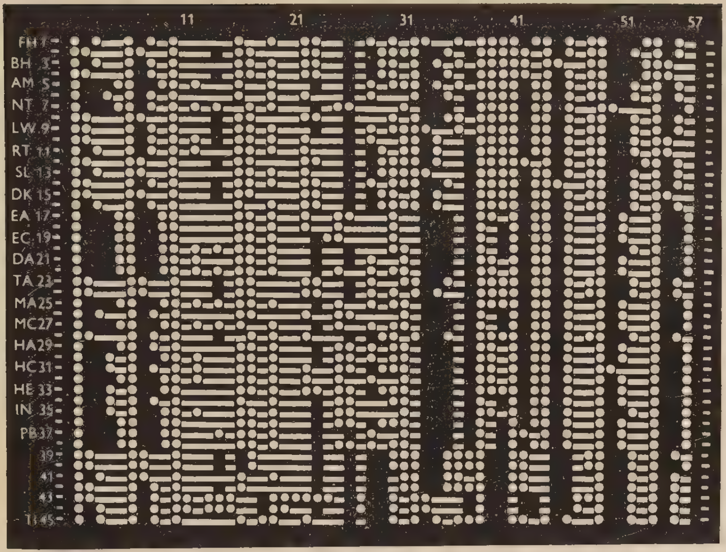
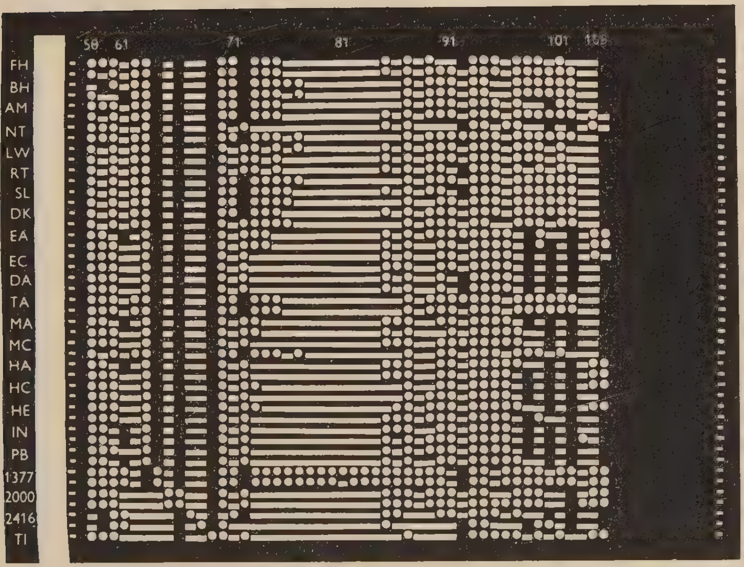

11
21
31
41
51
57
FH 1
BH 3
AM 5
NT 7
LW 9
RT 11
SL 13
DK 15
EA 17
EC 19
DA 21
TA 23
MA 25
MC 27
HA 29
HC 31
HE 33
IN 35
PB 37
39
41
43
TI 45

Fig. 2


58
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2000
2416
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Fig. 3

Figs. 2, 3. The table of strains versus features ($i \times m$ table) used for the worked example when printed on to X-ray film. The strains are tabulated in the rows (only alternate stains are lettered in) and the features in the columns. *Symbols*: positive value = circle, negative value = rectangle, NC value = blank, orientation marks = small dashes. Fig. 2. Features 1–57. Fig. 3. Features 58–105.

Ability to oxidize glucose in aerobic Hugh & Leifson medium within 7 days. Feature 72(A). When feature 71 is present it obscures the result and feature 72 is scored NC.

Definite acid production in peptone water carbohydrate media within 14 days. Features 73–84(A) for the carbohydrates fructose, mannose, trehalose, sucrose, starch, lactose, dulcitol, sorbitol, adonitol, melezitose, salicin and xylose respectively

Bulky rennin clot in litmus milk within 7 days. Feature 85(A).

Bleaching of the litmus in litmus milk within 7 days. Feature 86(A).

Table 3. *Part of the $i \times i$ table of n_s values*

		Strains					
		1	2	3	29	30	31
Strains	1	46	—	—	—	—	—
	2	37	49	—	—	—	—
	3	37	39	49	—	—	—
	29	26	25	22	40	—	—
	30	25	25	21	37	41	—
	31	26	27	25	34	36	41

Table 4. *Part of the $i \times i$ table of n_d values*

		Strains					
		1	2	3	29	30	31
Strains	1	0	—	—	—	—	—
	2	15	0	—	—	—	—
	3	15	14	0	—	—	—
	29	26	26	31	0	—	—
	30	27	26	32	5	0	—
	31	26	24	27	10	7	0

Production of definite ammonia in ammonia test. Feature 87(A).

Production of definite H_2S in 7 days. Feature 88(A).

Methylene blue reduction positive. Feature 89(A).

Nitrate reduction: detectable nitrite within 5 days. Feature 90(A).

Nitrite destruction. Features 91–92(C). Divided into three classes, no destruction, weak destruction and rapid destruction.

Catalase positive. Feature 93(A).

Phosphatase test, Features 94–95(C). Divided into negative, weak positive and strong positive.

Hydrolysis of aesculin detected in liquid medium within 6 days. Feature 96(A).

Casein hydrolysis in plate test after 2 days. Features 97–98(C). Divided into negative, narrow and wide zones of digestion.

Haemolysis of horse blood agar after 2 days. Features 99–100(C). Divided into negative, narrow and wide zones of haemolysis.

Egg-yolk reaction. Features 101–102(C). Divided into negative, narrow and wide zones of opacity after 2 days.

Malonate utilization test positive in 2 days. Feature 103(A).

Gluconate test after 4 days. Features 104–105(C). Divided into negative, weak positive and strong positive on heating with Benedict's solution.

The data after coding into features were entered into an $i \times m$ table on squared paper, and they were analysed by two methods, an electronic computer and by a rapid manual method. This latter technique, although laborious, is suitable for small numbers of strains, and is described since it may be useful to some workers. Each positive value is covered with a disk of adhesive black paper; each negative value is covered with a rectangle of black paper covering the lower half of the square. NC entries are left blank. Strain numbers are marked in black ink with orientation marks at the ends of each row. After erasing pencil marks the table is printed photographically on to film (X-ray film of 35 × 43 cm. size is suitable) and two copies prepared. The table is shown in Figs. 2 and 3 in this modified form, and will enable the subsequent working to be checked. When the duplicate copies are placed over one another on an illuminated background, one can orientate one row on any other row, and count the number of transparent circles (where two positive values coincide) and the number of semi-circles (where a positive and a negative entry coincide), thus obtaining n_s and n_d. Wherever NC occurs, it blots out the coincident symbol. The subsequent working is the same as that given below where an electronic computer was used.

Table 5. *Part of the $i \times i$ table of percent S values*

		Strains					
		1	2	3	29	30	31
Strains	1	100	—	—	—	—	—
	2	71·2	100	—	—	—	—
	3	71·2	73·6	100	—	—	—
	29	50·0	49·0	41·5	100	—	—
	30	48·1	49·0	39·6	88·1	100	—
	31	50·0	52·9	48·1	77·3	83·7	100

Table 6. *Part of the S table of strain pairs*

% S	Strain pairs	% S	Strain pairs
88·1	29.30	50·0	1.29, 1.31
83·7	30.31	49·0	2.29, 2.30
77·3	29.31	48·1	1.30, 3.31
73·6	2.3	41·5	3.29
71·2	1.2, 1.3	39·6	3.20
52·9	2.31		

The computer used was the Elliott 405 digital computer (Elliott Brothers, Boreham Wood, Herts). The computer programme for steps 1 to 9 is Bacterial Classification Mark I in the Elliott 405 computer library of programmes, and a copy of this programme is also deposited in the Science Museum Library, South Kensington, London, S.W. 7.

The computer worked through the steps given on p. 208. Some of the re-

Table 7. *The full table of the S values (%) from the worked example after re-arranging the strains by the sorting procedure*

Strain letters	Strain numbers	Strain numbers																																												
		2	15	24	23	8	12	13	14	3	4	16	28	6	5	9	11	10	1	32	21	22	27	25	29	38	36	30	35	20	31	37	33	17	18	19	34	26	7	39	40	41	42	45	43	44
MK	2	100																																												
DK	15	93	100																																											
TB	24	88	92	100																																										
TA	23	88	84	92	100																																									
TV	8	85	85	90	91	100																																								
LG	12	90	86	86	83	83	100																																							
SL	13	86	84	81	81	78	83	100																																						
RV	14	84	80	84	85	80	82	82	100																																					
BH	3	74	77	76	68	75	83	74	77	100																																				
BN	4	82	82	78	75	79	84	73	78	88	100																																			
MH	16	79	78	78	75	72	80	82	82	78	76	100																																		
PT	28	78	78	78	75	79	77	77	82	72	80	76	100																																	
MW	6	80	80	81	81	76	72	80	81	68	75	77	78	100																																
AM	5	73	73	80	77	75	71	73	75	67	66	76	69	75	100																															
LW	9	77	75	78	75	76	73	77	77	67	71	77	80	75	75	100																														
RT	11	73	77	68	71	65	69	80	69	67	67	78	70	74	69	76	100																													
SH	10	75	78	78	75	67	70	75	75	65	69	77	74	78	73	86	89	100																												
FH	1	71	73	76	73	75	68	77	72	71	69	75	72	77	74	78	69	71	100																											
HD	32	50	50	54	57	50	49	52	52	43	46	52	49	53	63	56	56	64	49	100																										
DA	21	48	48	50	53	46	43	52	50	38	41	47	46	50	60	56	60	61	50	87	100																									
NC	22	49	49	51	51	47	45	52	51	40	42	47	47	48	59	57	56	58	50	85	92	100																								
MC	27	47	47	49	51	47	43	48	48	37	41	48	45	48	61	55	53	56	49	77	80	88	100																							
MA	25	52	52	54	53	49	48	53	55	41	48	50	50	50	62	60	58	61	51	79	81	88	86	100																						
HA	29	49	49	51	50	46	45	50	53	42	47	50	49	51	56	59	54	51	50	74	77	79	79	81	100																					
RU	38	50	50	52	51	47	46	51	52	40	46	49	48	49	61	58	52	56	49	78	82	83	83	83	90	100																				
CA	36	52	52	50	49	47	48	53	47	42	48	51	47	47	56	53	54	54	47	78	77	78	74	79	81	89	100																			
HB	30	49	49	51	50	46	45	50	51	40	42	48	47	52	58	57	54	58	48	81	85	85	81	84	88	83	79	100																		
IN	35	48	48	48	50	46	42	49	48	39	42	47	46	50	55	53	56	59	43	77	81	78	74	80	80	79	77	88	100																	
GA	20	52	52	52	51	50	46	52	52	39	43	50	50	48	60	58	56	60	46	76	82	85	85	85	76	80	78	83	85	100																
HC	31	53	53	52	53	51	52	55	56	48	51	52	57	55	57	57	61	61	50	78	72	74	67	73	77	71	72	84	76	71	100															
PB	37	54	50	52	54	51	50	50	49	43	47	48	45	51	58	59	54	60	48	82	84	82	77	78	73	83	83	76	72	81	64	100														
HE	33	60	60	61	59	58	58	58	58	53	56	62	56	57	62	60	61	60	55	76	68	70	72	76	80	77	77	83	73	70	83	70	100													
EA	17	54	57	57	56	58	50	57	52	47	53	54	54	57	56	56	56	57	56	73	70	71	66	72	76	75	77	75	71	67	74	68	76	100												
EB	18	55	58	58	55	56	51	56	55	48	51	54	57	58	56	56	55	58	55	74	71	74	71	75	73	76	75	76	68	74	75	71	75	88	100											
EC	19	47	50	50	46	48	54	48	47	41	43	46	48	50	53	53	49	51	47	76	79	80	76	76	76	82	80	79	73	80	70	77	71	75	85	100										
HF	34	51	51	51	50	49	47	52	50	42	46	56	49	55	54	61	56	58	54	71	70	71	76	72	79	77	80	81	77	77	73	74	77	74	74	75	100									
MB	26	53	52	52	54	50	48	53	51	43	44	52	50	51	50	55	61	60	53	71	69	67	67	67	75	77	71	70	70	67	75	69	73	68	73	70	73	100								
NT	7	43	43	43	47	42	40	46	45	38	42	46	46	47	50	51	56	56	47	65	72	73	69	68	71	68	64	69	68	70	69	65	64	60	60	66	62	70	100							
NCTC 1377	39	62	55	57	59	61	60	59	56	54	54	53	53	52	51	55	47	48	50	34	36	38	37	39	42	38	38	41	37	41	39	40	44	44	43	36	41	38	36	100						
NCTC 9493	40	63	58	58	60	62	61	60	52	56	57	51	52	52	50	54	47	48	48	36	37	40	40	42	44	40	40	44	40	43	41	42	46	45	44	38	43	39	39	95	100					
NCTC 2000	41	57	49	51	55	53	49	54	50	49	47	58	47	56	53	60	53	55	53	50	50	47	52	48	56	50	50	56	54	55	49	58	56	50	51	49	59	49	47	54	50	100				
NCTC 6749	42	58	50	52	56	53	50	47	52	44	46	52	49	56	50	60	51	57	50	51	51	49	55	50	59	51	55	57	55	57	52	60	52	51	52	50	63	54	46	57	55	83	100			
TI	45	49	49	42	49	52	49	53	51	50	47	58	55	54	55	56	58	55	62	51	51	51	52	46	57	52	48	53	48	52	59	51	56	56	59	54	56	56	51	45	43	60	63	100		
GR	43	43	42	42	38	39	38	39	44	36	38	38	44	42	38	43	35	39	41	28	28	31	38	33	36	32	29	33	32	38	32	32	35	29	31	34	35	31	30	33	32	38	40	38	100	
TE	44	34	33	33	30	31	29	39	36	33	31	36	35	35	30	38	34	32	40	24	29	33	32	30	30	29	28	29	28	35	28	31	25	28	31	30	34	30	35	29	30	39	35	42	63	100

sults in the intermediate steps are given below. Part of the n_s and n_d values are tabulated in Tables 3 and 4, illustrating the results on three mesophilic strains (1, 2 and 3) and three psychrophilic strains (29, 30 and 31) of *Chromobacterium*. The figures for n_s for strains compared with themselves (i.e. the number of positive features possessed by the strains) seem to be characteristic of the group to which the strains belong. In Table 3 one can see that these figures are higher for strains 1–3 than for strains 29–31, and this was true generally for the other strains of the two groups. The S values were then calculated from the relation $S = n_s/(n_s + n_d)$.

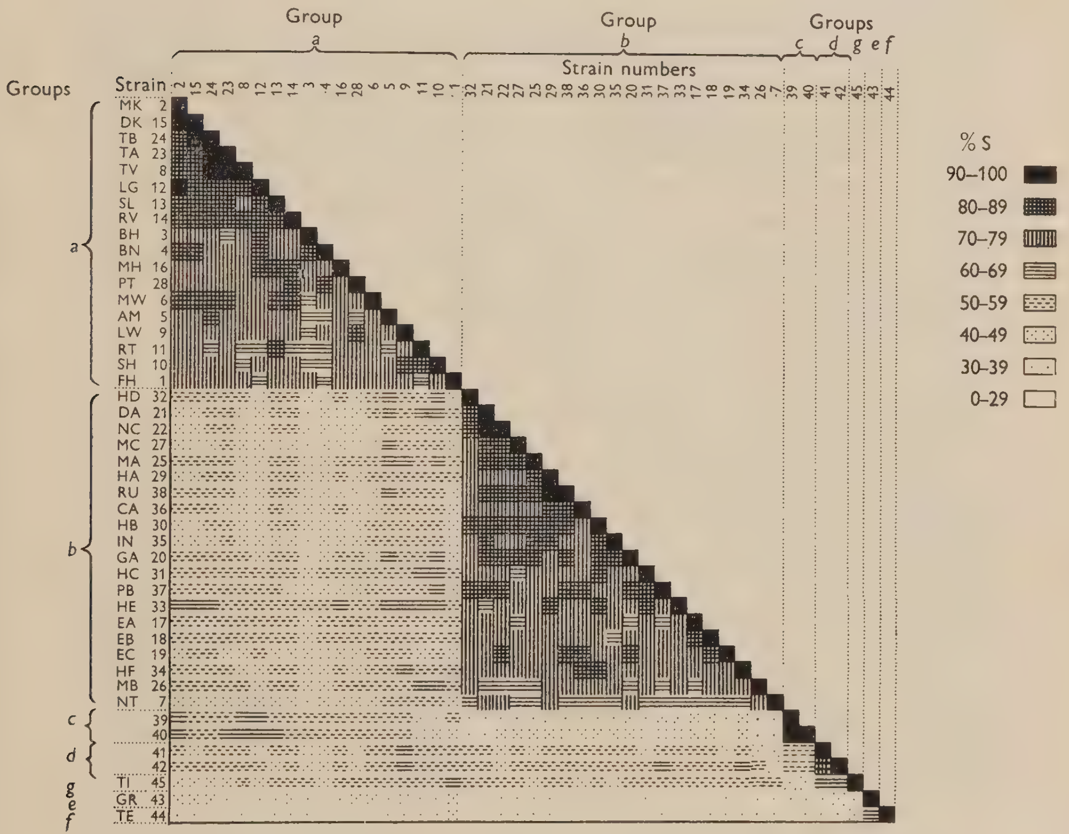
Group a
Group b
Groups c d g e f
Strain numbers
Groups
Strain
2 15 24 23 8 12 13 14 3 4 16 28 6 5 9 11 10 1 32 21 22 27 25 29 38 36 30 35 20 31 37 33 17 18 19 34 26 7 39 40 41 42 45 43 44
MK 2
DK 15
TB 24
TA 23
TV 8
LG 12
SL 13
RV 14
BH 3
BN 4
MH 16
PT 28
MW 6
AM 5
LW 9
RT 11
SH 10
FH 1
HD 32
DA 21
NC 22
MC 27
MA 25
HA 29
RU 38
CA 36
HB 30
IN 35
GA 20
HC 31
PB 37
HE 33
EA 17
EB 18
EC 19
HF 34
MB 26
NT 7
39
40
41
42
TI 45
GR 43
TE 44
a
b
c
d
g
e
f
% S
90–100
80–89
70–79
60–69
50–59
40–49
30–39
0–29

Fig. 4. Diagrammatic representation of the full S-value table of the worked example. The diagram is derived from Table 7 by shading the squares. The strains 39, 40, 41 and 42 are, respectively, NCTC 1377, NCTC 9493, NCTC 2000 and NCTC 6749.

The strains were then sorted into groups and the S table was rearranged in order to bring strains of a group adjacent to one another (steps 5–8). This may be done manually by the following procedure: the $i \times i$ table of S values is converted into an S table of strain pairs. Tables 5 and 6 show this for strains 1, 2, 3, 29, 30 and 31. It is seen that high values of S are found when a mesophilic strain is compared with a mesophilic strain, or when two psychrophils are compared. For these six strains the sorting procedure yields the results:

% S							
88		29,	30				
83	29,	30,	31				
73	29,	30,	31		2,	3	
71	29,	30,	31		1,	2,	3
52	29,	30,	31,	1,	2,	3	

This shows the separation of these strains into two groups at $S=71$ and the fusion of the groups at $S=52$.

The result of the sorting is shown diagrammatically in Fig. 1, in which it is seen that the expected groups of bacteria have been correctly formed. The table of S values was re-arranged to give the order of strains in Fig. 1, and this full table is shown in Table 7, to the nearest 1 % (the uncertainties of scoring the data do not justify greater accuracy). In this table the strain letters used in the text and in Sneath (1956*b*) are added to the strain numbers used in the analysis for ease of comparison. The full table is needed to check the results of the sorting procedure, since this might occasionally fuse two groups too soon or too late owing to an abberrant S value, and it is also needed for calculation of D values and of mean S values (step 9 and Table 2).

Overall similarity is a multidimensional quantity (one dimension for each feature) which has been expressed in three dimensions when given as an $i \times i$ table of S values. Therefore only a small amount of condensation can be made without losing information. This is because the inherent restrictions are few: thus if $S_{1.2}$ is 90 % and $S_{1.3}$ is 90 % then $S_{2.3}$ cannot be below 80 % provided there are no NC entries. But with NC entries and with more than a few strains this restriction becomes of little practical interest. It is possible without losing information to convert an $i \times i$ S value table into a three-dimensional model, made of rods whose length is proportional to S, and this may be useful on occasion. It can also be shown as an $i \times i$ table whose squares are shaded to represent the approximate S value, and the table from this example is shown in Fig. 4; the division between the groups is strikingly evident, together with some of the finer distinctions between one group and another.

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The Surface Structure and Serological Typing of *Bacillus megaterium*

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SUMMARY: The surface structures and serological typing of 54 strains of *Bacillus megaterium* were examined and compared with strain M. Morphologically the strains could be divided into 2 groups on the basis of cell size, fat content and susceptibility to enzymes. Forty homologous immune sera were prepared in rabbits. On the basis of the capsular and cell-wall reactions, 38 distinct serological types were found. The arrangement of the polysaccharide and polypeptide material in the capsules is described. Agglutination reactions confirmed the heterogeneity of the polysaccharides in the cell wall and capsules of *B. megaterium* and demonstrated a further relationship between 3 of the strains, probably due to the flagellar antigen. Precipitin reactions carried out with the supernatant fluid from broth cultures or with cell-wall extracts showed a much broader specificity.

An unusual complex capsular structure showing a topographically distinct localization of D-glutamic acid polypeptide and a type-specific polysaccharide was first demonstrated in strain M of *Bacillus megaterium* (Tomcsik, 1951), designated in previous studies as *Bacillus* 'M'. The discovery that lysozyme induces a spherical transformation of the protoplasts of this strain led to the demonstration of the specific cell-wall reaction (Tomcsik & Guex-Holzer, 1952). It was also shown that, contrary to previous observations, the cytoplasm of these bacilli could be digested with trypsin. The resulting cytoskeleton consists mainly of the cell wall and also gives a microscopically visible specific reaction with the homologous immune serum (Tomcsik & Guex-Holzer, 1954*a*, *b*; Tomcsik & Grace, 1955; Tomcsik & Baumann-Grace, 1957). These trypsin-treated bacteria, when inoculated into rabbits, produced antibodies which reacted with the cell wall and with the polysaccharide components of the capsule but not with the isolated protoplasts. In contrast, antibodies produced by the isolated protoplasts reacted with the thermolabile trypsin-sensitive component of the bacterial surface as well as with the protoplasts themselves, but serologically were completely distinct from the cell wall and capsule (Tomcsik & Guex-Holzer, 1954*b*). Thus *Bacillus megaterium* strain M proved to be exceptionally well-suited to enzymic and serological investigation as well as to the study of antibodies as cytological indicators (Tomcsik, 1956). These results obtained with *B. megaterium* strain M showed that several cytologically-definable elements of the cell structure, namely the two capsular materials, the thermolabile antigen of non-capsulated bacteria, the cell wall and the isolated protoplasts could be used for serological typing. Although some of the observations described above were confirmed with one or two authentic strains of *B. megaterium*, their application to the species as a whole was unknown. We have attempted, here, therefore, a serological study of the surface structures of *B. megaterium*.

METHODS

Organisms. Forty-five strains of *Bacillus megaterium* were isolated from soil and examined together with the 'M' strain (Tomcsik, 1949) and 9 laboratory strains received from Dr J. P. Aubert, Institut Pasteur, Paris (1), Dr C. Weibull, Stockholm (KM strain), Dr S. Raffel, Stanford University, California (2) and Dr Ruth Gordon, Rutgers University, New Brunswick (5).

Isolation of organisms. Ten g. soil were shaken with 100 ml. sterile physiological saline. After standing to allow the large particles to sediment, 2 ml. of the cloudy supernatant fluid were removed and heated at 80° for 20 min. After cooling, 2 loopfuls were spread over the surface of a plate of Gladstone & Fildes CCY agar (Gladstone & Fildes, 1940) and incubated 20–24 hr. at 28°. Organisms from the various colony types were examined with a phase-contrast microscope and those characteristic of *Bacillus megaterium* and *B. cereus* purified by plating.

Identification of strains. Bacillus megaterium strains were identified and separated from *B. cereus* according to the data given by Smith, Gordon & Clark (1952), using mannitol fermentation, the V.P. test and egg-yolk reaction (L.V.; McGaughey & Chu, 1948) as the main criteria. All the soil strains produced spores at least on isolation and their morphology was noted. In addition, the ability of the strains to produce polypeptide capsules was examined.

Media. All strains were maintained on Gladstone & Fildes CCY agar. Liquid cultures were obtained in Gladstone & Fildes CCY broth either by aeration or by shaking in the T tubes described by Kay & Fildes (1950).

Fat staining. The fat content of the organisms was judged after staining with Sudan Black B, by the method described by Burdon (1946). Carbol fuchsin (1/40) was substituted for safranin in the counterstaining.

Phase-contrast preparations were made as described by Tomcsik & Guex-Holzer (1954*a*).

Immune sera. The D-glutamic acid polypeptide antibody was that described previously (Tomcsik & Guex-Holzer, 1954*a*, *b*). The homologous antibodies against whole organisms, cell walls or protoplasts of the 'M' and other strains of *B. megaterium* were produced by the intravenous inoculation of rabbits with 6–10 doses of the suspensions at 3- to 4-day intervals. The animals were bled 10 days after the last injection.

Agglutination reactions. The mixtures of antigen and antibody were incubated at 37° for 10 min., then centrifuged for 4 min. and the test read immediately after shaking up the sediment.

Precipitation reactions. Ring precipitation reactions were carried out with the supernatant fluid obtained after centrifuging 14 hr. T-tube shake cultures, or with extracts of trypsin-digested bacteria (see below).

Trypsin digestion. Whole organisms from agar or broth cultures were suspended in physiological saline and the density adjusted to give a reading of *c.* 180 on the Klett Summerson photoelectric colorimeter, with blue filter no. 42. Three vol. of organisms were added to 1 vol. of 1% (w/v) trypsin

(Siegfried, Zofingen) in saline. The pH value was adjusted to 8·2 with phenolphthalein and 2 % (w/v) Na_2CO_3; chloroform was added as preservative. After 14–20 hr. incubation, the cell remains were centrifuged and resuspended in saline. Extracts for the precipitation reaction were prepared as follows: the trypsin-digested organisms were supended in either 1/8000 crystalline lysozyme (Armour) in saline, 1 % (w/v) sodium taurocholate in distilled water or in 0·25 N-HCl. The digestions were allowed to proceed for 1 hr. at 37° (lysozyme at room temperature) after which the cell-wall remains were removed by centrifugation and the supernatant fluid used in the precipitation reaction. The hydrochloric acid extract was neutralized before testing.

Lysozyme digestion was carried out by the methods described by Baumann-Grace & Tomcsik (1957) with crystalline lysozyme (Armour).

RESULTS

Characteristics of the Bacillus megaterium *strains*

Both the freshly-isolated and the laboratory strains corresponded to the descriptions given by Smith *et al.* (1952) and gave mostly typical biochemical reactions, although 3 strains (1 laboratory strain and 2 isolates) did not ferment mannitol even after continued subculture on the medium. The strains could be broadly divided into two morphological groups. Those designated as group A were large, *c.* 1·2–1·5 μ. or more in diameter and contained large amounts of fat, often filling the entire organism; about 43 % of the isolates belonged to this type. In contrast, those strains allocated to group B possessed usually rather narrower cells, *c.* 0·9–1·2 μ. in diameter, and contained very little lipid material which was usually concentrated at the edge of the organism or at the cross-septa giving the appearance of a cell-wall stain with Sudan Black B. Only occasionally were small discrete globules detected in the phase-contrast microscope or after staining. Contrary to the recommendation of Smith *et al.* (1952) the use of glucose agar did not significantly increase the amount of fat in most of the group B strains. In addition groups A and B differed in their sensitivity towards lysozyme and trypsin, group B strains being on the whole considerably more readily digested by both enzymes than group A strains (Tomcsik & Baumann-Grace, 1956; Baumann-Grace & Tomcsik, 1957). Sporulation was observed in all our isolates from soil. Two morphologically interesting strains were found. In one laboratory strain, the culture consisted of very long filaments in which very few cross-walls could be detected, and in one isolate abnormal division with longitudinal cross-septa was occasionally observed.

Serological reactions of strain M

Twenty rabbits were immunized with 8–10 intravenous injections of living organisms of strain M and the serological reactions of the best (M 15) and three average sera (M 3, M 4, M 16) from this series are summarized in Table 1. All 20 immune sera elicited the specific capsular reaction. On addition of serum to the capsulated organisms of strain M obtained from agar cultures, the capsular transverse septa and the polar caps were rendered visible immediately and the margin of the capsule was indicated by a weak radial striation (Pl. 1, fig. 1). The antibody eliciting this reaction could be absorbed with isolated capsular mucopolysaccharide (Guex-Holzer & Tomcsik, 1956). Application of antiserum obtained by the immunization of rabbits with heat-killed *Bacillus anthracis*, on the other hand, reacted only with the polypeptide part of the capsule of strain M organisms, revealing a homogeneous structure. The gaps expected on the place of the polysaccharide transverse septa were not usually visible in the phase-contrast microscope, probably because of their comparatively small size; only by modifying the cultural conditions was it possible to demonstrate a striated structure (Tomcsik & Guex-Holzer, 1954*a*). The fact that none of the homologous anti-M sera contained polypeptide antibody, whereas the latter is produced in at least 50 % of the immune sera prepared against *B. anthracis*, suggests that in *B. megaterium* the D-glutamic acid polypeptide occurs only in the haptenic form, in contrast to *B. anthracis*.

Non-capsulated cells of strain M were obtained from an 8–20 hr. aerated or shaken broth culture in which there was little chain formation and the single organisms were usually motile. A specific cell-wall reaction could be elicited on these organisms after trypsin digestion; the titre was usually similar to that of the specific capsular reaction.

Table 1. *Serological reactions of anti-M immune sera produced in rabbits against whole bacteria, cell walls and protoplasts*

No. of immune serum	Immune serum against	Specific capsular reaction	Specific cell-wall reaction	Agglutinin titre of sera against		
				living bacteria	cell walls	protoplasts
M3	Living bacteria	+ + +	+ + +	1280	240	n.e.
M4	Living bacteria	+ + +	+ + +	640	120	n.e.
M15	Living bacteria	+ + + +	+ + + +	2560	640	320
M16	Living bacteria	+ + +	+ + +	960	120	n.e.
M14	Cell walls	+	+ +	160	80	neg.
M31	Cell walls	+ +	+ +	640	320	1:20
M32	Cell walls	+ +	+ +	320	160	1:20
M33	Cell walls	+ + +	+ + +	640	320	1:20
M23	Protoplasts	–	–	320	neg.	160
M26	Protoplasts	–	–	640	neg.	320
M27	Protoplasts	–	–	640	neg.	320
M28	Protoplasts	–	–	1280	neg.	640

– = no reaction with undiluted serum; +, + +, + + +, + + + + = visible reactions with 1/2, 1/5, 1/10, or 1/20 diluted serum; neg. = no agglutination in 1/20 serum dilution; n.e. = not examined.

All the immune sera prepared against trypsin-treated organisms and protoplasts of strain M are included in Table 1. Specific cell-wall or capsular reaction was not elicited by the protoplast sera, showing that these entities are serologically distinct. The non-capsulated living bacteria were, on the other hand, agglutinated in varying degree by all three types of immune sera. Further examination of the reaction of the protoplast antibodies and cell-wall antibodies with whole bacteria revealed that two components were involved in the agglutination, differing from one another in their sensitivity towards trypsin. The protoplast immune sera agglutinated the intact, non-capsulated bacteria because of a thermolabile trypsin-sensitive component on the surface of the latter which was easily removed in 15–30 min. by simple shaking. There is evidence to suggest that this antigen is probably flagellar material; it is interesting that it is still present on the intact protoplasts after complete digestion of the cell wall with lysozyme in isotonic sucrose solution (Weibull, 1953). On the basis of the data summarized in Table 1, it is not possible to distinguish between the antibodies which react with the flagella and those which react with the protoplast proper. These results show that the thermolabile surface antigen as well as the thermostable capsular and cell-wall polysaccharides must be taken into consideration in a serological study of the various types of *Bacillus megaterium.*

Serological typing of Bacillus megaterium, *based on the specific capsular and cell-wall reactions*

The typing of the remaining 54 strains of *Bacillus megaterium* was carried out as follows. Immune sera to five selected strains of *B. megaterium* were prepared by the immunization of rabbits with whole organisms, using one rabbit for each strain. The resulting sera were used to carry out the specific capsular and/or cell-wall reaction on each of the 55 strains. Strains giving even a slight reaction were eliminated and, from those remaining, 5 were selected for immunization and the tests repeated. In this way homologous sera against 40 strains were prepared with which a total of 2255 cross-reactions for the phase-contrast study of the specific capsular reaction were performed. On the basis of these cross-reactions further similarities were found in 3 pairs of strains; all the remaining immune sera apparently contained separate type antibodies. Thus, including strain M, 37 different serological type antisera were obtained which gave a specific capsular reaction with 51 of the 55 strains. More than 300 specific cell-wall reactions were carried out crosswise, which demonstrated the serological parallelism between the specific cell-wall reaction and the capsular reaction which was observed in strain M. With the help of the specific cell-wall reaction (Pl. 1, figs. 2, 3) it was possible to demonstrate the relationship between two hitherto untyped strains which gave no capsular reaction because of loss of capsule. Thus, with the help of both reactions, 38 distinct serological types were obtained. It should, however, be emphasized that some of the cross-reactions were weak, indicating that some of the strains were similar but not identical; when these are omitted, the number of distinct types is even higher.

Tabulation of the serological reactions of all the strains would require too much space and only a few of the more interesting cross-reactions may be mentioned. Two serologically identical strains were of geographically distant origin, one being isolated in the U.S.A. and one in Switzerland. Four of the laboratory strains contained serologically similar capsular and cell-wall substances. One of these strains was obtained from the Pasteur Institute, Paris, two from Dr Raffel, Stanford University (one of which was designated originally as *Bacillus tumescens*) and one from Dr Weibull (Stockholm). The last mentioned strain KM has been used in most of the protoplast studies (Weibull, 1956; McQuillen, 1956) and is uncapsulated, but it gives with the immune sera prepared against the 'Paris' strain, as well as with the serum against one of our own isolates, a strong specific cell-wall reaction. Considering the large number of serological types found in *B. megaterium* it seems possible therefore that these four old laboratory strains giving similar specific capsular and cell-wall reactions have a common origin.

Since so many serological types were found, it was of interest to examine the typical complex capsular structure to see whether the capsule characteristic of strain M occurred with any regularity in the other strains and whether other morphological arrangements of the polysaccharide and polypeptide are to be found. A capsular structure, entirely similar to that described in strain M was found in 10 of the strains of *Bacillus megaterium* (Pl. 1, fig. 1). Morphologically all resembled strain M and belonged to group B (the narrower cells). Repeated examinations of these 10 strains revealed always the same regular arrangment of the capsular polysaccharides. In a few cases, however, abundant production of capsular polypeptide prevented the diffusion of the polysaccharide antibody and caused a capsular refraction. Four of these strains together with strain M were examined by Dr C. Weibull (personal communication) with regard to their cytochrome systems, and were found to be identical. The arrangement of the capsular polysaccharide in 12 other strains was similar but less typical. Transverse septa were found only on a few division levels; more frequently a thickening of the polysaccharide occurred at the points of division and at the poles, giving a bamboo-like appearance to the chains. Only 7 of these strains were 'narrow-celled' and belonged to group B. One of these was also examined by Dr Weibull with regard to its cytochrome system and was found to be identical with that of strain M. Five strains belonging to morphological group A showed a distinct comb-like structure with homologous antiserum (Pl. 2, fig. 4*a*, *b*). The units of this structure were similar in size and showed no additional thickening at the division level. The remainder of the strains possessed a thin, almost homogeneous polysaccharide capsule on the cell wall, and in some cases it was possible to demonstrate that it was surrounded by a layer of polypeptide in such a way that the capsular substances were concentrically arranged (Pl. 2, figs. 5, 6).

Application of *Bacillus anthracis* polypeptide antiserum elicited a polypeptide capsular reaction in 53 of the 55 *B. megaterium* strains. Two old laboratory cultures no longer gave a polypeptide reaction. All 45 strains obtained from soil possessed a well-defined polypeptide capsule shortly after isolation, in contrast to the remaining old laboratory strains in which only a thin polypeptide layer was detected. It was observed that several of the freshly isolated strains lost much of this material after continued transfer on agar medium, which suggests that polypeptide production is a characteristic of strains of *B. megaterium* living in soil. The polypeptide capsule more often appears homogeneous than striated, even in 13 strains of group B which showed typical capsular transverse septa with homologous serum. Only 14 of the 53 strains gave a comb-like reaction.

The possibility that a third material existed in the capsule was demonstrated in one strain in which the polysaccharide antibody of strain M elicited a marginal reaction of the capsule whereas addition of homologous antibody demonstrated only the interior layer (Pl. 2, figs. 7, 8). Polypeptide antibody reacted with the exterior region of the capsule.

Serological typing of Bacillus megaterium, *based on the agglutination reaction*

In previous studies on strain M of *Bacillus megaterium* it was observed that large capsules tended to inhibit the agglutination reaction, and for this reason the reactions summarized in Table 1 were carried out with non-capsulated bacilli. With strain M the latter could always be obtained from aerated or shaken broth cultures, but unfortunately these methods of cultivation did not totally prevent capsule formation in most of the other strains. However, satisfactory agglutination reactions were obtained with 41 of the sera which with the homologous strains gave titres ranging from 1/160 to 1/2500. The non-specific agglutination titre was found to be 1/10 or less. In order to simplify the technique of carrying out cross-reactions between 41 sera and 55 strains, the preliminary agglutination tests were performed with a serum dilution of 1/100, thus eliminating some of the weaker side-reactions. The same major cross-reactions were disclosed by the agglutination test as were observed by the specific capsular and cell-wall reactions. Only one additional cross-reaction was observed. Strain M showed hardly any relationship to the other *B. megaterium* strains in the morphological reactions, but cross-reacted with one strain (Mg 50) and showed a more distant relationship to a second (Mg 49) in the agglutination tests. The observations leading to the elucidation of these reactions are summarized in Table 2.

Table 2. *Serological relationship of the* Bacillus megaterium *strains M, Mg* 49 *and Mg* 50

Immune sera against	Strain	Specific capsular reactions	Specific cell-wall reactions	Agglutination titre of sera against living bacteria	trypsin digested bacteria	shaken bacteria
Strain M	M	++	++	160	160	160
Cell wall	Mg 49	±	–	.	.	.
	Mg 50	–	–	.	.	.
Protoplast	M	–	–	1280	.	.
	Mg 49	–	–	.	.	.
	Mg 50	–	–	1280	.	.
Strain Mg 49	M	±	–	160	.	.
whole bact.	Mg 49	+++	+++	640	320	320
	Mg 50	–	–	160	.	.
Strain Mg 50	M	±	–	2560	.	.
whole bact.	Mg 49	–	–	.	.	.
	Mg 50	++	+++	2560	1280	1280

± = very weak marginal reaction. Other signs see Table 1.

Morphological examination confirmed that neither the cell wall nor the capsule were involved in the cross-reactions. The antibody obtained with the cell wall of strain M was strictly specific, but the antibody produced from the protoplasts of this strain cross-agglutinated intact bacteria of strain Mg 50 to full titre. Since trypsin digestion or simple shaking for an hour without glass beads abolished the cross-reaction, it was concluded that the 'protoplast antibody' probably reacted with flagellar material.

The serological specificity of substances released into the medium in fluid cultures

Precipitation reactions were carried out between the culture supernatant fluid of 55 strains and 41 immune sera. The uninoculated medium did not give a precipitation reaction with any of the sera. Contrary to the sharp specificity of the capsular and cell-wall reactions, the culture supernatant fluids of most of the *Bacillus megaterium* strains gave an unexpectedly large number of cross-reactions. Only type-specific reactions were obtained with trypsin-treated bacteria in the specific cell-wall and agglutination reactions. However, after further treatment of these bacteria with lysozyme, sodium taurocholate (1 %, w/v) or 0·25 N-HCl the extracts showed the same broad specificity in the precipitation reaction as the culture fluids.

DISCUSSION

The primary purpose of this work was to analyse the surface structures and the serological typing of 55 strains of *Bacillus megaterium*. Morphological and cytochemical observations showed that the strains of this species may be broadly divided into two groups: group A, those characterized by broad cells and large amounts of fat inclusions; group B, the smaller-celled strains possessing hardly any fat globules. Previous observations (Baumann-Grace & Tomcsik, 1957) suggested that differences in the cell-wall structure between the two groups exist, since most of the strains belonging to group A could not be completely digested with lysozyme whereas those of group B were rapidly and easily dissolved. However, biochemical differences between the two groups were not observed and polypeptide was produced by all strains on isolation, in contradistinction to *B. cereus* (details of this observation will be published elsewhere).

On the basis of the specific capsular reaction, with antisera prepared in rabbits against 40 of the strains, 37 distinct serological types were found. Previous work (Tomcsik & Guex-Holzer, 1951, 1954*a*) had shown that these type differences may be assumed to be due to specific polysaccharides. In 11 strains, the homologous sera revealed a capsular structure similar to that originally described in strain M, characterized chiefly by thin cross-septa occurring as a continuation of the cross-walls, and polar caps. These strains belonged entirely to group B. A similar but less typical arrangement was found in 12 other strains. The remainder of the strains (most of which belonged to group A) showed various morphological arrangements of the capsule on the addition of homologous antiserum, the most commonly occurring form showing radial striation. A number of strains, particularly those from old laboratory cultures, showed only a thin homogeneous polysaccharide layer surrounding the bacilli and the exterior layer of the capsule could only be detected by applying polypeptide antibody. In those strains which showed striations or transverse septa on addition of polysaccharide antibody, the complementary arrangement of the polypeptide capsular material could only be demonstrated when the polysaccharide 'discs' were sufficiently thick.

The specific cell-wall reaction performed with trypsin-treated bacteria completely confirmed the type distribution found by the capsular reaction. Essentially similar results were also obtained by the normal agglutination technique, thus stressing the major role played by the cell wall in the serological relationship of *Bacillus megaterium.* In the few discrepancies observed with the agglutination reaction, it was found that the cell walls were serologically distinct and that the cross-agglutination was probably due to the similarity of the flagellar antigen. The problem why antibodies against the flagella are formed when a protoplast suspension deprived of cell-wall material is used for immunization, remains to be solved.

The results of the specific capsular and cell-wall reactions, as well as of the agglutination tests, justify the conclusion that the intact capsules and cell walls of *Bacillus megaterium* exhibit a very large number of serological types, greater, in fact, than any other species studied up to the present. Thus it is surprising to find that the bacteria-free culture fluids from 15 hr. cultures or extracts of cell walls showed a much broader specificity, giving rise to many more cross-reactions. The reason for this is not clear, but it may be suggested that either the autolytic and chemical breakdown products of the capsular and cell-wall polysaccharides are less specific or that these processes liberate polysaccharides with a broader specificity from the deeper layers of the cell wall and which are normally not involved in the specific morphological and agglutination reactions.

The use of serological typing in the identification of vegetative *Bacillus megaterium* is not practicable owing to the large number of serological types encountered. In fact, as many as five distinct types were isolated from one single soil sample. The possession of a capsule which contains both polypeptide and polysaccharide material showing a distinct morphological arrangement seems to be a characteristic of the species and might be used in classification. For identification purposes, however, the strains should be examined for capsule production soon after isolation, as experience has shown that this character tends to diminish or to be completely lost on continued subculture.

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REFERENCES

Baumann-Grace, J. B. & Tomcsik, J. (1957). Lysozym-Empfindlichkeit der Species *B. megaterium*. *Experientia*, **13**, 148.

Burdon, K. L. (1946). Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *J. Bact.* **52**, 665.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Guex-Holzer, S. & Tomcsik, J. (1956). The isolation and chemical nature of capsular and cell-wall haptens in a *Bacillus* species. *J. gen. Microbiol.* **14**, 14.

Kay, D. & Fildes, P. (1950). The calcium requirements of a typhoid bacteriophage. *Brit. J. exp. Path.* **31**, 338.

McGaughey, C. A. & Chu, H. P. (1948). The egg-yolk reaction of aerobic sporing bacilli. *J. gen. Microbiol.* **2**, 334.

McQuillen, K. (1956). Capabilities of bacterial protoplasts in *Bacterial Anatomy. Symp. Soc. gen. Microbiol.*, **6**, 126.

Smith, N. R., Gordon, R. E. & Clark, F. E. (1952). Aerobic sporeforming bacteria. *Agric. Mongr.* no. 16.

Tomcsik, J. (1949). Zur Frage der induzierten Mutation der Milzbrandbazillen. *Schweiz. Z. Path.* **12**, 498.

Tomcsik, J. (1951). Complex structure of the bacterial capsule in the genus *Bacillus*. *Experientia*, **7**, 459.

Tomcsik, J. (1956). Antibodies as indicators for bacterial surface structures. *Annu. Rev. Microbiol.* **10**, 213.

Tomcsik, J. & Grace, J. B. (1955). The specific cell-wall reaction and staining of the bacterial cell wall with Alcian blue. *J. gen. Microbiol.* **13**, 105.

Tomcsik, J. & Baumann-Grace, J. B. (1956). Zellwandfreie Bakterienprotoplasten. *Verh. naturf. Ges. Basel.* **67**, 218.

Tomcsik, J. & Baumann-Grace, J. B. (1957). Wirkung des Trypsins auf die Bakterienzellstruktur. *Schweiz. Z. Path.* **20**, 129.

Tomcsik, J. & Guex-Holzer, S. (1951). Anthrax-Polypeptid und andere spezies-spezifische Substanzen der Kapsel in der Bazillus-Gruppe. *Schweiz. Z. Path.* **14**, 515.

Tomcsik, J. & Guex-Holzer, S. (1952). Änderung der Struktur der Bakterienzelle im Verlauf der Lysozym-Einwirkung. *Schweiz. Z. Path.* **15**, 517.

Tomcsik, J. & Guex-Holzer, S. (1954*a*). A specific cell-wall reaction in *Bacillus* sp. *J. gen. Microbiol.* **10**, 317.

Tomcsik, J. & Guex-Holzer, S. (1954*b*). Antikörperproduktion mit isolierter Bakterienzellwand und mit Protoplasten. *Experientia*, **10**, 484.

Weibull, C. (1953). The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. *J. Bact.* **66**, 688.

Weibull, C. (1956). Bacterial protoplasts; their formation and characteristics in *Bacterial Anatomy. Symp. Soc. gen. Microbiol.*, **6**, 111.

EXPLANATION OF PLATES

All figures: bacteria from Gladstone–Fildes agar cultures examined by phase-contrast microscopy at × 2000.

Plate 1

Fig. 1. Strain M after addition of homologous immune serum.

Fig. 2. *B. megaterium* strain No. 1 after treatment for 7 hr. with 1/400 trypsin. The cell wall is faintly visible without antibody.

Fig. 3. The same microscopical field as in fig. 2 after the addition of homologous antibody.

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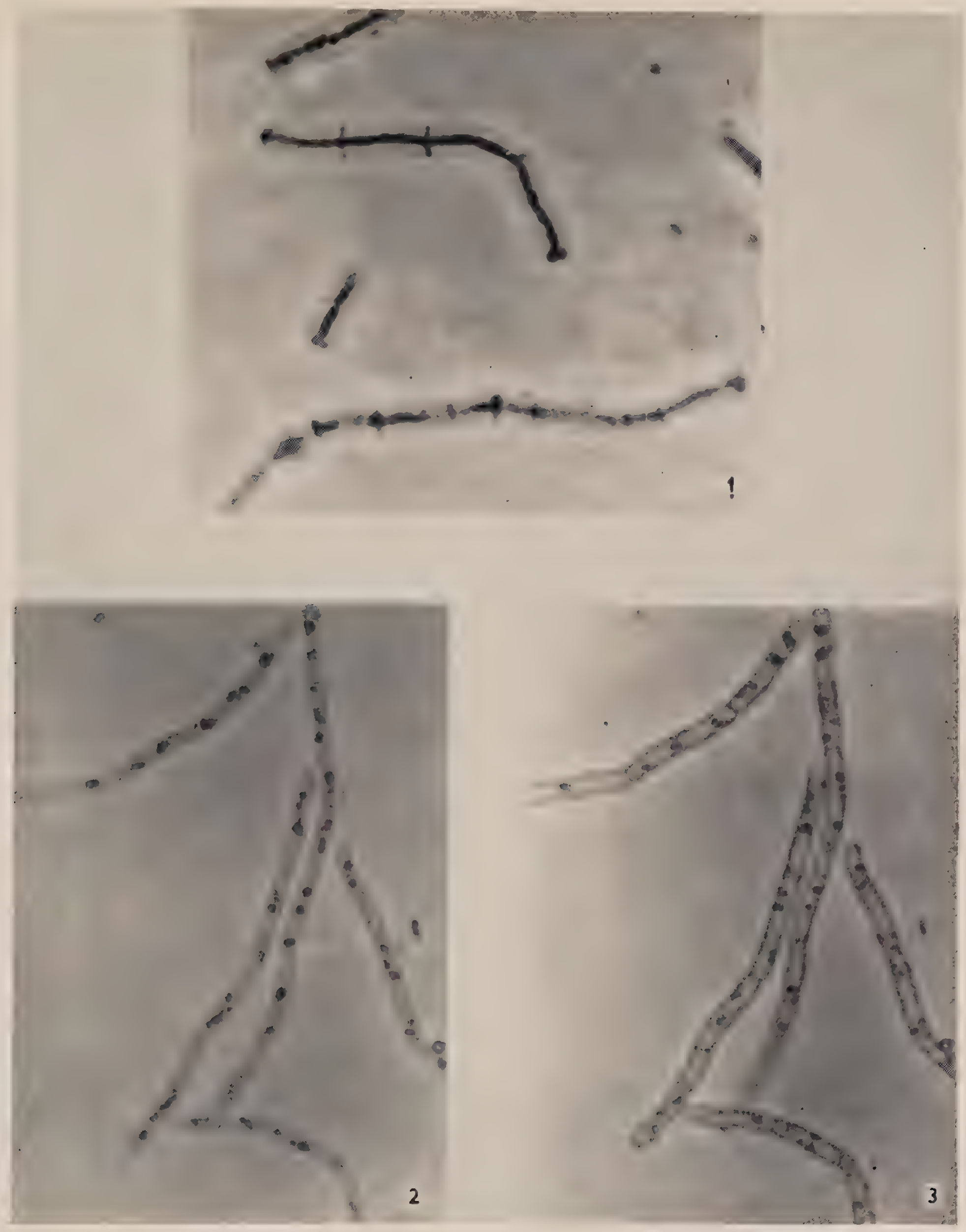
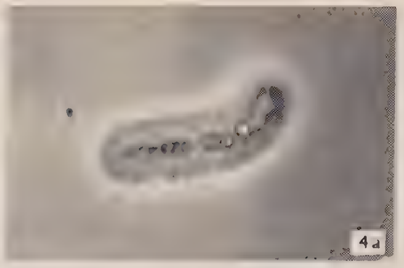
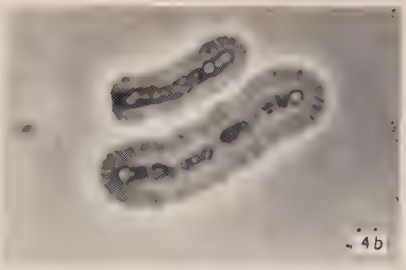
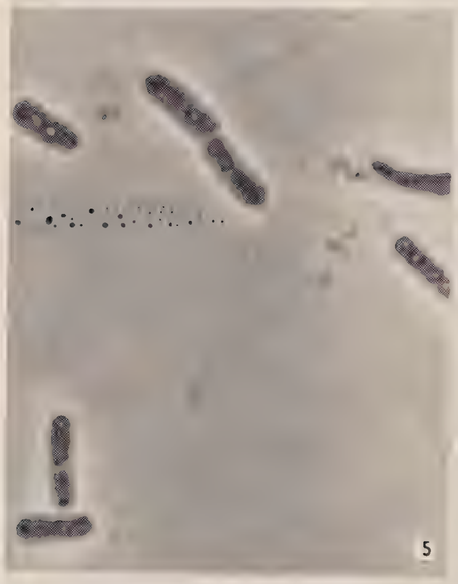


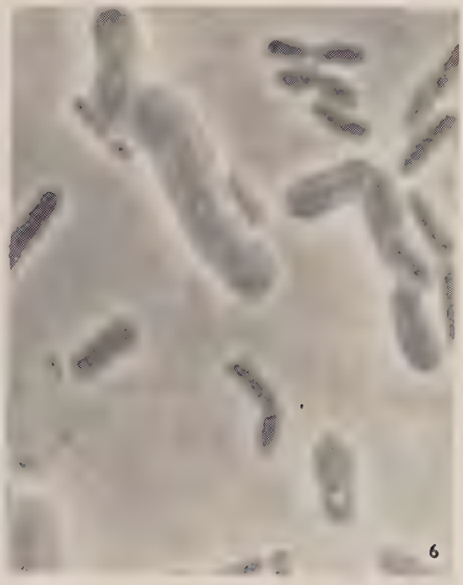
J. B. BAUMANN-GRACE AND J. TOMCSIK—THE SURFACE OF *B. MEGATERIUM*. PLATE 1

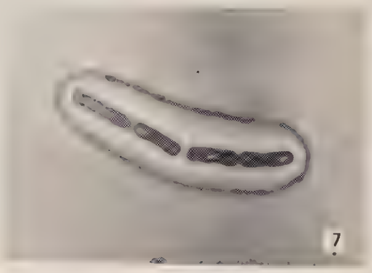
(*Facing p.* 236)

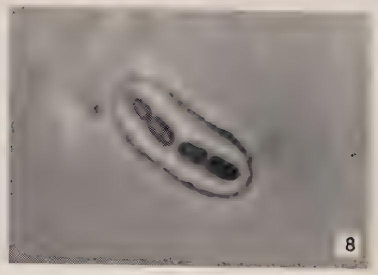












J. B. BAUMANN-GRACE AND J. TOMCSIK—THE SURFACE OF *B. MEGATERIUM*. PLATE 2

PLATE 2

Fig. 4*a*, *b*. *B. megaterium* strain no. 21 after addition of homologous immune serum. A comb-like capsular structure is revealed.

Fig. 5. *B. megaterium* strain no. 2 together with the homologous antibody; the interior layer of the capsule is revealed.

Fig. 6. The same preparation as in fig. 5, after successive addition of anthrax-polypeptide antibody. The exterior layer of the capsule is now revealed.

Fig. 7. *B. megaterium* strain no. 49 after addition of strain M anti-serum. The heterologous antibody reacts only with the surface layer of the capsule.

Fig. 8. The same preparation as in fig. 7, after addition of the homologous immune serum. The interior layer of the capsule reacts with the homologous antibody.

(*Received* 11 *March* 1957)

CORRIGENDUM

In Rogers, H. J. (1957), *J. gen. Microbiol.* **16**, 22–37.

On p. 30, abscissa to Fig. 3:

for: 'ΔG (mg. bacterial-N/ml.)'
read: 'ΔG (mg. bacterial-N/ml. $\times$ 10)'

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Lwoff, A. (1957). *J. gen. Microbiol.* **17**, 239–253

The Concept of Virus

The Third Marjory Stephenson Memorial Lecture

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> *Things thought too long can be no longer thought,*
> *For beauty dies of beauty, worth of worth,*
> *And ancient lineaments are blotted out.*
>
> William Butler Yeats: The Gyres (*Last Poems,* 1936–39)

From the letter inviting me to give this third Marjory Stephenson Memorial Lecture I understood that the subject should be 'general'. Most scientists, as you know, are maniacs. One of my manias is to consult dictionaries. The dictionary says that general means 'completely or approximately universal', but it fortunately adds 'within implied limits'. I decided that the limits of universality would be the borderline of the kingdom of viruses.

The notion of virus, the subject of this lecture, is a *pons asinorum* of microbiologists, and I suspect that some of you have come to see how I behave on the asses' bridge. It seems therefore only fair that I should take the maximum of risks. Thus I have decided not to avoid discussing the notion of organism and the notion of life, which are considered to be highly treacherous subjects.

The man in the street generally considers viruses as the dangerous agents of infectious diseases. If one has to lecture before an assembly of microbiologists, one becomes aware of the fact that the real danger lies with the virologists. When by reading their writings one tries to understand what a virus is, one reaches a sort of feeling of the possible existence of some slight theoretical misunderstandings amongst virologists in which it may be dangerous to be involved. Some virologists are convinced that viruses are micro-organisms. This view is expressed in Sir MacFarlane Burnet's book *Virus as Organism.* Other virologists, like Wendell Stanley, feel that viruses should be considered as molecules. A third class is represented by F. C. Bawden and N. W. Pirie who write, 'statements that viruses are small organisms should be regarded with as much suspicion as statements that they are simply molecules'. If one wants to know who is right, one needs only to read Dr C. H. Andrewes's article *Viruses as Organisms*: 'It is my experience', writes Dr Andrewes, 'that those who have studied viruses from the widest point of view are more apt to consider them as organisms, whereas others who have looked on them from perhaps a more restricted point of view are more apt to toy with other hypotheses.'

In the light of these remarks the negative conclusion of Bawden & Pirie might appear as the result of the absence of any view, whether wide or restricted. Being however in complete agreement with them, I am inclined to consider that this opinion is the result of concentrated thinking.

If a virus be neither organism nor molecule, what is its nature? What is a virus? It is a malady of our time that words are often deprived of their meaning. Many people like to think that a virus is something different from a virus.

My ambition is to show that the word virus has a meaning, and I shall defend a paradoxical viewpoint, namely that *viruses are viruses*. As simplistic as this statement might appear to some of you, the task is not an easy one. It will be necessary to analyse critically the behaviour and properties of viruses at various phases of their life cycle, and to find out the nature of the difference between viruses and other infectious agents, between viruses and micro-organisms. Then, if we have landed somewhere, we have to decide where we have landed. A brief historical survey is here a necessity.

History

Until the end of the nineteenth century the history of viruses is just a part of the history of infectious diseases. In about 2500 B.C. the Chinese had identified small-pox and knew that it was transmissible. To write however without comment, as some people do, that the Chinese knew viral diseases is perhaps not entirely justified. Aristotle was aware of the fact that rabies was transmitted by the bite of dogs, and the Hebrews used to compare this bite to that of a venomous snake. In latin *virus* means 'venom' or similar poisonous fluid. A virus was something which could produce a disease. And in A.D. 50, Cornelius Aulus Celsus produced this remarkable sentence: 'Rabies is caused by a virus.' The ideas of Celsus about viruses, considered in the light of our present knowledge, were probably rather primitive and therefore Celsus should not be considered as the real founder of virology.

Ideas concerning infectious diseases remained metaphysical until the concept of a specific agent emerged. This was mainly the result of the study of epidemics of syphilis which in this respect, as well as in the development of antibacterial chemotherapy, has been a highly beneficial disease.

Then, mainly as a consequence of Pasteur's work, the agents of infectious diseases were identified as microbes. These agents whether bacteria, protozoa or fungi, were called viruses.

Pasteur and Roux had no difficulty in proving that rabies was a specific infectious disease. Although they were unable to see the agent, they quite naturally considered it to be a small microbe. When Iwanowsky discovered that the juice of tobacco plants showing the symptoms of mosaic disease remained infectious after filtration, he also concluded that the infectious agent was a small microbe. Then came Beijerinck who confirmed the filterability of tobacco mosaic virus. He also discovered that the infectious power was not lost by precipitation with ethanol and that the infectious agent could diffuse through agar gels. The infection, wrote Beijerinck, is not caused by microbes but by a fluid infectious principle. This intuition of genius about a difference of nature between tobacco mosaic virus and micro-organisms makes Beijerinck the real founder of conceptual virology.

Beijerinck's views were so opposed to the current ideas that they did not receive any attention. This did not prevent the discovery of a number of infectious filterable agents which were considered to be small microbes and which were therefore called filterable viruses or ultraviruses. Then someone remarked that, because ultraviruses were small microbes, they should be called inframicrobes. For obvious reasons, none of the scientists studying filterable infectious agents was pretentious or modest enough to describe himself as an ultra-virologist or as an infra-microbiologist. And as everybody has to be labelled, these scientists were labelled as virologists. Quite naturally, as a result of the principle of the least effort, the ultraviruses, the filterable invisible infectious agents studied by virologists, became viruses. And as a counter-stroke the microbes, the visible infectious agents, were deprived of their ancestral right to be called viruses. This swing produced a confusional state from which microbiology has not yet recovered. One of our problems will be to find out whether or not the disease is curable.

The bacteriophage

A general lecture should stand high above technical details and should therefore not include any data. The notion of virus, however general it may be, nevertheless has to be founded on a few facts. And I hope to be forgiven if I remind you briefly of the life cycle of a bacteriophage.

A bacteriophage is a nucleoproteinic particle adorned with a tail. When the tip of the tail meets a receptive bacterium it attaches itself to the bacterial cortex. Then the genetic material of the phage, that is to say its nucleic acid, passes into the bacterial cytoplasm. The infected bacterium can then evolve in two ways.

(1) The genetic material of the phage multiplies and specific phage proteins are synthesized. This is the vegetative phase, at the end of which some 100 phage particles are organized. They will be liberated as a result of the lysis of the bacterium.

(2) The genetic material of the phage does not multiply. It reaches a specific locus or receptor of the bacterial chromosome and attaches itself to it. By virtue of this attachment, the properties of the genetic material of the phage are modified and it is now a prophage. The prophage behaves as if it were a bacterial gene. It divides together with the bacterial chromosome and at the division is transmitted to each of the daughter bacteria. From time to time, either for unknown reasons or as the result of the action of mutagenic-carcinogenic agents, the prophage loses its connexion with the chromosome, starts the vegetative phase of the cycle, and phage particles are produced. Bacteria which carry a prophage are called lysogenic. They are endowed with the property of producing phage in the absence of infection. The prophage is the substratum of this property. It is the structure which carries the information necessary for the production of phage particles.

We have now to discuss a few features and properties of bacteriophage. As rightly emphasized by Luria, the ability to be introduced from outside is a requirement for the recognition of a virus as such. This is, by the way, one of the few theoretical points on which all virologists agree. If infectiousness is eliminated from the definition of viruses then we are no longer able to discriminate between viruses and the cellular organelles endowed with genetic continuity. Now we can perform a test and ask a few questions of a certain number of people.

Table 1. *The temperate phage*

The genetic material of the phage or germ may be in three states: proviral genome; vegetative genome; infective genome.

Phase of the life cycle	Constitution	Activity	Infectivity	Pathogenicity as such
Proviral	Nucleic acid (NA);	Replication of nucleic acid	0	0
Vegetative	Nucleic acid + proteins of phage;	Replication of nucleic acid. Synthesis of phage proteins	0	+
Infective	Nucleoprotein;	Nil	+	·

If one asks whether the prophage or the vegetative phage is a virus, the answer is no. If one asks whether the phage particle is a virus, the answer is yes. The reason is simple. Infectivity being considered as a characteristic trait of viruses, something is recognized as a virus only when it possesses this feature. And we deny the right of being called viruses to those phases of the life cycle which are devoid of this character. This is exactly as if one would deny the right of being a Plasmodium to those phases of the life cycle of the malarial parasite which are not infectious for the mammal. Allow me here a short digression.

Mammals are said to be characterized by the possession of mammae which secrete milk for the nourishment of the young. And it is true that mammals produce milk. It is also true that a number of us who will never produce any milk are nevertheless mammals. This remark might seem irrelevant. It has however a profound meaning which reaches far beyond mammals and viruses. It means that the most distinctive characteristics of a class are not necessarily present, are not necessarily expressed, in all the individual members of the class at each phase of their life cycle.

In more general terms it means that the properties of a category of a given order are transcended by the properties of a category of a higher order. This is of the utmost importance when discussing the definition of a phage. Reproduction is a necessary feature of bacteriophage. If one denies to the vegetative phage the right to be called a phage because it is not infectious, one should not call the bacteriophage particle a virus because it does not reproduce itself. This is obviously absurd.

It is evident that a bacteriophage is neither this nor that but is necessarily the sum of the various phases of its life cycle. Any definition of a bacteriophage should be an integration. When one considers the life cycle, one sees first that the only structure which is common to all the three phases is the genetic material. One sees also that the infectious phage particle never multiplies as such, but is produced by the organization of phage material. An infectious particle is never the direct descendant of another infectious particle. Ultimately, as already seen, some of the phases of the cycle are not infectious.

Table 2. *The bacteriophage as a whole*

	Prophage	Vegetative phage	Phage particle
Specific nucleic acid	+	+	+
Replication of nucleic acid	+	+	0
Synthesis of proteins; pathogenicity; morphogenesis of particles	0	+	0
Infectivity	0	0	+

A definition of the phage should therefore not be centred on the infectious particle. The following definition is proposed: *a bacteriophage is a strict parasite of bacteria possessing an infectious phase and which is multiplied in the form of its genetic material and which is sometimes pathogenic.* According to this definition, the prophage and the vegetative phage are parts of the 'bacteriophage' as well as the infectious particle which is thus deprived of its supremacy.

The notion of bacteriophage should not be transcended by the notions of infection and of disease, which are only particular aspects of the bacteriophage considered as a whole.

Infection

By considering phage with more attention the conclusion will be reached that it is not only the supremacy of the infectious particle which is on trial but the notion of infection itself.

According to the Oxford Dictionary, *infectious* means: *having the quality or power of communicating disease by infection.* And infection is defined as the *communication of a disease.* Let us consider a few examples.

A typhoid bacillus infects an animal which dies of typhoid. The agent of the disease is the infectious bacterium. There is no problem. If one injects spores of tetanus bacilli into an animal, the spore is infectious, but the active agent is the bacterium which is inside the spore. A mosquito carrying *Plasmodium malariae* gives malaria to an animal. The mosquito is infectious, but it is clear that the agent of the disease is not the mosquito, but the malarial parasite.

A bacteriophage particle injects its genetic material into a bacterium which dies. The phage particle is infectious, but the agent of the disease is the genetic material of the phage which has started the vegetative phase. A male lysogenic bacterium copulates with a non-lysogenic female. The male bacterium injects its chromosome and its prophage into the female which dies. The male lysogenic bacterium is infectious, but the agent of the disease is the prophage. Thus, the genetic material of the phage, which is by itself not infectious, is the agent of the infectious disease. What is more disturbing is that the penetration of the genetic material of the phage is not necessarily followed by a disease. The bacterium can be lysogenized as the result of an 'infection' and remain healthy. Should the penetration of the genetic material be described as an infection or not depending on the fate of the bacterium?

When scrutinizing all the cases of 'infection' it becomes manifest that the essence of infection is not the disease, but *the introduction into an organism of a foreign entity able to multiply, to produce a disease, and to reproduce infectious entities.*

This definition eliminates all the pitfalls and excludes from infectiousness all the normal cell structures which can penetrate into another cell, such as the transforming principle, the lethal genes, and the gene which controls the synthesis of bacteriocins. It is clear that one has to discriminate between the thing which infects or injects and the object which is injected. The foreign entity, which penetrates into, and reproduces in, an organism as a result of an infection is a sort of *infectum.* When dealing with bacteria, the *infectum* is the bacterium itself. When dealing with bacteriophage, the *infectum* is its genetic material.

This reasoning will probably be considered as specious and sophisticated and I shall be accused of rigorism, as if strictness were not simply exactitude and precision.

It is well known that we are unable to think without mental schemes. It is therefore better to know what these schemes are. Because bacteriophage is the agent of an infectious disease, we have applied to it the scheme and terminology derived from the study of infectious diseases caused by microorganisms, and, as a result, our ideas have been distorted.

Viruses in general

So far, we have considered bacteriophage as a model of virus. We should now consider viruses in general. An important remark is necessary here. Mammals are defined as animals able to secrete milk. It appears immediately that this definition has a value only if other animals exist which do not secrete milk. Whatever the importance of a class, its definition must not only include some objects, but exclude other objects. The presence of a character as a part of the definition of a class has its necessary counterpart in its correlative absence in another class. The concept of mammals has a meaning because of the existence of animals such as reptiles or birds which do not secrete milk, which are not mammals.

We speak of viruses as different from bacteria, protozoa, fungi and algae. This implies the existence of a category of infectious agents, viruses, which are different from the other infectious agents. As we aim to be scientists, we have to state clearly the nature of the difference.

Viruses are often opposed to bacteria because of their size. A virus, according to some virologists, should have at least one dimension lower than 200 mμ. If dimensions have any meaning it is not by the astrological virtue of a number, but because of a correlation between size and some essential properties which are responsible for fundamental differences.

In order to find out the essence of this difference, let us examine and compare the bacteriophage considered as a model of virus on the one hand, and on the other hand, a typical micro-organism such as protozoon, a yeast or a bacterium.

(1) All typical micro-organisms contain both types of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Bacteriophage contains only one type, DNA.

Table 3. *Organisms, viruses and cellular constituents*

			Cellular constituents	
	Organisms	Viruses	Genetic material	Organelles endowed with genetic continuity
Types of nucleic acid	2§	1	1	?
Multiplying as nucleic acid and produced from nucleic acid only	0	+	+	0
Growth and division*	+	0	0	+
Presence of Lipmann system	+	0	0	0 (+)†
Infectivity	+	+	0	0

* Concerns micro-organisms only. The replication of a structure by the template mechanism is neither considered as growth nor as division but as replication.

† Some organelles only contain a Lipmann system.

§ 2=DNA+RNA, 1=DNA or RNA.

(2) All typical micro-organisms are reproduced from the integrated sum of their constituent parts, nucleus, cytoplasm, cytoplasmic structures, cortex. Bacteriophage is produced or reproduced from its nucleic acid only. When considering a micro-organism, the *infectum* is the micro-organism itself; when considering a bacteriophage, the *infectum* is its genetic material.

(3) The essence of the multiplication of any cell or micro-organism is the replication of the genetic material, but multiplication is not separable from growth which is the result of the synthesis of cell material. During the growth of a micro-organism, the individuality of the whole is maintained. Growth culminates in binary fission. Binary fission has no meaning at the molecular level. An individual molecule cannot undergo fission; the replication of nucleic acid by a template mechanism is not a binary fission. Binary fission has a meaning only when we consider either a complex organelle or structure or an organism.

The essence of phage multiplication is also the replication of its nucleic acid. During the vegetative phase, there is an increase of phage material, but the nucleic acid and the proteins, so far as we know, do not form an organized structure. What increases is a disorderly mixture of phage constituents. There is nothing which could divide. There is no binary fission in a bacteriophage.

(4) Micro-organisms possess a system of enzymes which convert the potential energy of foodstuffs into the high energy bonds which are needed

for biological syntheses, including patternization. This system, which might be called the 'Lipmann system', is part of the micro-organism itself. The Lipmann system is absent from bacteriophage. The energy for the synthesis and patternization of phage material is provided by the host cell.

Numerous small infectious agents, whether they are parasites of plants or animals, possess a number of characters which are present in bacteriophage. They possess only one nucleic acid; they multiply in the form of their nucleic acid; they can be reproduced from their nucleic acid; they are unable to grow and to undergo binary fission; they depend for their energy on the Lipmann system of the host cell.

Viruses could accordingly be defined as: *strictly intracellular and potentially pathogenic entities with an infectious phase*, and (1) possessing only one type of nucleic acid, (2) multiplying in the form of their genetic material, (3) unable to grow and to undergo binary fission, (4) devoid of a Lipmann system. It should be noted that any one of characters 1 to 4 is sufficient to identify an entity possessing an infectious nuclear-proteinic phase as a virus, and it is quite possible that these features or attributes are correlated and subordinated.

If one wants to put emphasis on the infectious particles the following definition may be proposed: viruses are infectious, potentially pathogenic, nucleoproteinic entities possessing only one type of nucleic acid, which are reproduced from their genetic material, are unable to grow and to undergo binary fission, and are devoid of a Lipmann system.

As already seen, an object, a category or a concept, has an individuality or a reality only because of the existence of different objects, categories or concepts. The art of definition is founded on differences as well as on resemblances. A definition has to exclude as well as to include. Our definition of viruses is valid only because, at the same time, it includes a homogeneous class of entities, viruses, and excludes another homogeneous class of entities, micro-organisms.

We are therefore justified in uniting into a special class those entities called viruses. The concept of virus is thus established on a firm ground and the term virus has, at last, a definite meaning.

Are viruses organisms?

Small infectious agents have been subdivided into two categories: viruses and non-viruses, the latter being micro-organisms. Micro-organisms are organisms. Are viruses organisms?

Let us make here another mammalian digression. Mammals are obviously not reptiles, but they have evolved from a primitive reptile. Thanks to this common origin, mammals share with reptiles a certain number of traits, as for example the presence of vertebrae. Therefore they are united into a group of vertebrates which transcends the differences and exemplifies what is in common.

If viruses have evolved from micro-organisms, whether primitive or not, they might have kept one or many features of organisms. The existence of those common features would allow one to visualize viruses as a special class of micro-organisms, that is to say, to visualize viruses as organisms.

The first thing to do is to define organisms. Micro-organisms being organisms, it is plain that all the main characters which have been ascribed to micro-organisms are among the characters of an organism. These are: the presence of two types of nucleic acids (DNA and RNA), the ability to grow, the presence of the Lipmann system. These features are also present in the cells of multicellular organisms, the cell *stricto sensu*. Since it is obvious that a cell *stricto sensu* is not an organism, these features are not sufficient to define an organism.

An organism may be visualized as *an independent unit of integrated and interdependent structures and functions*. In a multicellular organism, the integrated and interdependent parts are the cells. We shall leave these multicellular organisms aside and consider only micro-organisms or protista. In protista, the integrated and interdependent parts are the cellular organelles or structures, such as the chromosomes, the mitochondria, the enzymes, the cortex, etc. Cellular organelles are not organisms because they are dependent, because they are parts of a whole which is the organism itself, the ultimate unit of independent reproduction.

One of the main tasks of an organism is to control its dependent and interdependent parts. Harmonious growth is the result of the balanced synthesis of cellular material and the balanced replication of various structures. We know to-day that harmonious growth is controlled by the interplay of metabolites and antimetabolites produced as the result of enzymic activity. Let us then consider critically the arguments which are generally put forward in order to justify the statement that viruses are organisms.

(1) 'Viruses multiply; they are reproduced true to type; they contain nucleoproteins'.

These characters are actually to be found in organisms. Unfortunately, they are to be found, either isolated or together, in a number of cellular organelles such as the nuclei, the mitochondria, the chloroplasts, the kinetosomes.

(2) 'Viruses are antigenically different from the host cell'.

In a cell each individual enzyme has its antigenic individuality. Such a thing as antigenicity of a cell in general does not exist.

(3) 'Viruses and bacteria form a regular gradient of size.'

This is true, but between a molecule and an elephant one finds entities of intermediary sizes and this does not prove that an elephant is a molecule or vice versa.

The rules of formal logic establish the validity of a syllogism. These rules have to be applied by virologists. In order that a character should be valid for the union of two classes into a common category, this character should be absent from other categories. For example, worms and vertebrates possess a digestive tract, but this does not prove that worms are vertebrates because insects also possess a digestive tract. Viruses and organisms have a few characters in common. These characters being also present in cellular organelles, they cannot be considered as supporting the conclusion that viruses are organisms. It is really strange that so many people take delight in looking straightforwardly at problems which they have so carefully posed askew.

Almost sixty years ago Beijerinck pointed out that the multiplication of tobacco mosaic virus has many points in common with the multiplication of amyloplasts and chloroplasts. Despite the fact, said Beijerinck, that both the virus and the plastids have an independent existence, their multiplication is controlled by cellular growth; to-day we would rather say by cellular metabolism. Bawden has reached the conclusion that: 'If we are to seek analogues for viruses, the closest may well lie among cellular components'. When one considers the essential properties of viruses it is clear that they have more in common with cellular organelles than with micro-organisms.

In a general article on the influenza virus which appeared in February 1957, one may read the following: 'a virus is not an individual organism in the ordinary sense of the term, but something which could almost be called a stream of biological patterns'. I should like to say that I am in complete agreement with this statement which, by the way, is due to Sir MacFarlane Burnet.

Are viruses alive?

The classical question as to whether viruses are or are not alive depends necessarily on our conception of life. Life may be considered as a property, a manifestation or a state of cells or organisms. This excludes from the living, all cellular particles or structures, whether or not endowed with genetic continuity. It is evident, as already seen, that the properties of an organism are more than the sum of the individual properties of its individual parts. An organism is the result of the integration of its dependent and interdependent parts. The essential character of an organism, independence, with all its implications, transcends the characters of its parts, dependence. Life is precisely this transcendence. Those who share this viewpoint will conclude that viruses are not alive.

The origin of viruses

Most of those who have discussed the problem of the origin of viruses have considered the virus particle. Let us instead consider briefly the problem of the origin of the prophage.

Two main theories have been proposed: (1) the prophage is the residue of the degradation of a parasitic bacterium or of a more or less primitive organism; (2) the prophage was born by genic or chromosomal mutations of the bacterium which thus became lysogenic. Instead of trying to find which theory is right, let us try to build a third theory which would suppress the contradiction.

Bacteria are a very homogeneous and certainly monophyletic group, which differ from all other protista by the organization of the mitochondrial apparatus. This means that *Escherichia coli*, for example, and the hypothetical parasitic bacterium which is supposed to have degenerated into a prophage of *E. coli* have a common ancestor. In other terms the genetic material of *E. coli* and the genetic material of the prophage have originated from the very same genetic material. And the common structure which has been preserved in the bacterial chromosome as a receptor, and in the prophage where it accounts for lysogenization, is the signature of the common origin.

If one admits that the prophage had its origin in the genetic material of a bacterium, it might have originated in any bacterium and especially in a more or less remote ancestor of the very bacterium which is now lysogenic. The phase at which evolution has taken place is not as important as the existence of a common origin. The endogeneous and exogeneous theories can be reconciled in the following way: the genetic material of the bacteriophage and the genetic material of the bacterium have evolved from a common structure, the genetic material of a primitive bacterium. Whatever the origin of the genetic material of the bacteriophage might have been, we know to-day that the prophage behaves as if it were a bacterial gene: its reproduction is controlled by the bacterium as a whole just as is the reproduction of any bacterial gene or bacterial structure. The prophage is obviously not independent. It behaves as a dependent part of an organism, but this organism is not the bacteriophage, it is the bacterium.

Between 1855 and 1870, microbiology was founded. It was established that micro-organisms were specific living beings which always came from an identical living being. An animal injected with a specific microbe shows a specific disease and the specific infective agent is reproduced true to type. This is true for bacteria and also for viruses. The theory of spontaneous generation or heterogenesis according to which a living organism could arise from anything or everything was wiped out.

A theory is a generalization and it tends to absorb as much as possible, especially those aberrant data which, in the purgatory of science, await an interpretation. The more truth a theory contains, the more it includes. The more it includes, the greater its power. The greater its power, the more dangerous it is. A discovery leads to a theory, a theory to a law, a law to a religion. According to the dogma, infectious diseases are caused by specific micro-organisms. Viral diseases being infectious, it was concluded that viruses were specific micro-organisms. And when the idea was proposed that viruses were perhaps not microbes but might have originated from some pathological constituents of their host cell, then the theory of spontaneous generation seemed to revive from its ashes. The supporters of the endogeneous theory were accused of heresy and a smell of sulphur was floating in the air. The inquisitors of faith have tried, and are still trying, to ridicule the endogenous theory by brandishing the threadbare scarecrow of heterogenesis. The endogenous theory of the origin of viruses is a theory. It may be wrong, but it is not absurd. If discussed at all, it should be discussed scientifically.

Those who claim to possess the truth should remember that heterogenesis was once 'the truth'. And the spectre of heterogenesis should be allowed to rest in the famed purple sheet where scientists shroud their dead gods.

When one walks in the country one sees cows, sheep, hogs, horses, men, one sees individual mammals. One never sees a mammal in general because such a thing does not exist. The mammal in general could be a dream, but it is a concept. And it is essential to remember that a concept is an idea of a class of objects. The virus is a concept. Our ideas concerning the origin of viruses may differ, and also our ideas of the place we assign to viruses among other entities. The very use of the term virus, however, implies the acceptance of viruses as a specific class of entities. The choice of a discriminative character of a class cannot be a matter of taste. Either a character offers itself indiscriminately to any category and is valueless, or it belongs exclusively to one category and is thereby discriminative, that is to say valid. A concept is the equivalent of a doctrinal corpus. It has to be sound and coherent and should stand firm on a strictly logical base.

The mammal in general could be a dream....

Autocriticism

Whilst giving birth in pains and tears to this concept of the virus I have kept a diary. It was a sort of analysis and criticism of the intellectual operations involved in the process. Autocriticism being nowadays rather fashionable I thought an abridged version of the diary would not be completely out of place as a manner of conclusion. Those who might feel sad at the idea of being deprived of the pleasure of heterocriticism may be reassured at once: I have left aside for their personal enjoyment all the really critical points.

So, what have I done? The data pertaining to viruses have been considered in themselves and then integrated, that is to say, united in order to form a whole. To cement the stones a mental element was introduced. The operation which has been performed is what Whewell called a colligation, a colligation being defined by the Master of Trinity as 'an operation by which facts are united under one and the same idea'. The word was new at the beginning of the nineteenth century, but the idea was an old one. It corresponds to the Aristotelian transition from particular to general, the transition to a superior degree of generality. It is closely related to Bacon's *Interpretatio naturae*, and also to induction which is the transition from facts to laws. I have not dealt with laws, but I have attempted to visualize the virus as a whole, to introduce a general idea, the notion or concept of virus. In doing so, I have tried to be as rationalistic and logical as possible.

I have been constantly aware of the fact that I had to submit my thoughts to the compatriots of John Stewart Mill whose empirical logic has exerted and still exerts a considerable influence in this country. It is customary to oppose British empiricism to French logic. Let us see what happens sometimes on the continent.

In his *Introduction à l'Etude de la Médecine Expérimentale*, Claude Bernard claimed that philosophers were without utility for scientists; that they loaded the mind of scientists with all sorts of inapplicable principles. The useful principles, said Claude Bernard, are those which emerge from the details of experimental practice. Claude Bernard, who was neither an empiricist nor a logician, may be designated as an experimentalist. And perhaps, in the last analysis, the difference between empiricism and logicism is not so marked in reality as in the brains of philosophers. It would seem that a number of scientists, although empiricists, produce perfectly valid concepts. This is really depressing, and philosophers cannot do much about these aberrant people except to describe them as unconscious colligators.

'Frenchmen', said Paul Valéry, 'deem possible, and even in conformity with the essence of things, that a prodigiously diverse ensemble of highly complex phenomena can be and must be condensed and finally reduced into a few plain formulae, at the same time necessary and sufficient.' Belonging to an hyperlogical extrovert nation, I have coined numerous definitions as if I had really penetrated the essence of things. And I should not have discussed the intimate nature of viruses with more confidence if I had been myself a virus.

'The French', said T. E. Lawrence, 'see by the directly-thrown light of reason and understanding, not through the half-closed eyes, mistily, by things' essential radiance, in the manner of the imaginative British.'

Some scientists visualize the virus as an ill-defined shape emerging bashfully out of a dense and golden cloud. This is a beautiful and romantic vision. Virology should, however, not be too Turnerian. Nor should it be an abstract art. The portrait of a virus should not produce an aesthetic emotion by means of an organic disturbance. The virus is amenable to intellectual analysis. I have cut its life cycle into slices and analysed each slice as lucidly as I could. The significance and value of each character has been dissected with a sort of sadism. This, I realize, is mere intellectual butchery, the type of job which it is advisable to leave to somebody else.

As a result, the virus has been unveiled. Now, it stands before you, naked. Do not turn your eyes away, it is only a concept. And the concept of virus, just as any other concept, the concept of a lecture, the concept of woman, is not as difficult to handle as the real object.

I have tried to make everything as clear, as perceptible and as ostensible as possible. The operations which have been performed were required for intellectual hygiene and intellectual comfort. And yet, I am afraid that in performing this task I have deprived myself of one of the most prized privileges of elegance, namely invisibility.

According to William James, to ignore, to disdain, to consider, to overlook, is the essence of the gentleman. I have considered, but I have not ignored, nor disdained, nor overlooked enough. Perhaps I have displayed a truly plebeian excess of logic and of passion. It would perhaps have been more aristocratic to look at things from a distance with a haughty and stylish detachment.

It is certainly pleasant to be a mammal. Yet, I am conscious of having produced such an excessive number of mammalian digressions that you might have had the impression that the lecture was dealing with the concept of mammal rather than with the concept of virus. Also, I have made use of the American word patternization. *Mea culpa. Mea culpa.* I confess my sins and hope they will be remitted. I have behaved improperly and as a punishment, the conclusion of this lecture will be prosy, coarse and vulgar: viruses should be considered as viruses because viruses are viruses.

APPENDIX

Remarks on the pathogenicity of viruses

Viruses are pathogenic or potentially pathogenic entities. The property of eliciting a disease is shared by a number of micro-organisms and by a few abnormal cellular organelles such as, for example, lethal genes. Bacteria, just as 'higher organisms', also exhibit diseases of metabolism. It is known, for example, that the synthesis of colicin, pyocin, megacin or in more general terms, of bacteriocins, is a fatal disease. These lethal biosyntheses are, so far as we know, controlled by genetic determinants. The originality of virus is that the malady initiated by the genetic material generally culminates in the morphogenesis of infectious particles.

The objection will naturally be raised that oncogenic viruses which are responsible for malignant growth do not kill their host cell. This is true, yet the oncogenic virus kills the organism. When one considers the pathogenic action of an ordinary virus such as bacteriophage, one sees that the virus kills its host cell because it disturbs the equilibrium of its dependent parts. If one analyses the pathogenic action of oncogenic viruses one realizes that they modify the host cell which is a dependent part of an organism. In general terms, when considering the organism which transcends its individual interdependent parts, we have the right to say that oncogenic viruses are pathogenic.

REFERENCES

ANDREWES, C. H. (1952). Viruses as organisms. In *Poliomyelitis*. Second int. Poliomyelitis Conf. pp. 3–5. Lippincott Co.

BAWDEN, F. C. (1952). Virus and its interaction with the host cell: biochemical aspects. In *Poliomyelitis*. Second int. Poliomyelitis Conf. pp. 9–12. Lippincott Co.

BAWDEN, F. C. & PIRIE, N. W. (1951). Virus multiplication considered as a form of protein synthesis. In *The Nature of Virus Multiplication*, Symp. Soc. gen. Microbiol. **2**, 21.

BEIJERINCK, M. W. (1898). Über ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter. *Verh. Akad. Wet. Amst.* **6**, fas. 5.

BURNET, F. M. (1950). *Virus as Organism*. Harvard University Press.

BURNET, F. M. (1957). The structure of the influenza virus. *Sci. Amer.* **196**, 37.

JACOB, F. & WOLLMAN, E. L. (1957). Genetic aspects of lysogeny. In *The Chemical Basis of Heredity*, p. 468. Edited by W. D. McElroy & H. B. Glass. Baltimore: The Johns Hopkins Press.

LURIA, S. E. (1953). *General Virology*. New York: John Wiley and Sons.

LWOFF, A. (1953). Lysogeny. *Bact. Rev.* **17**, 269.

LWOFF, A. (1954). *Control and Interrelations of Metabolic and Viral Diseases of Bacteria*. The Harvey Lectures, series L, Academic Press, 92.

LWOFF, A. (1957). Cell and Organism. *Proc. N.Y. Acad. Sci.* **5**, (in the Press).

LWOFF, A. (1957). Nutrition and metabolism in collateral fields. *Cancer Res.* (in the Press).

STANLEY, W. M. (1952). Viruses as chemical agents. In *Poliomyelitis*, pp. 6–8. Second int. Poliomyelitis Conf. Lippincott Co.

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Gorrill, R. H. (1957). *J. gen. Microbiol.* **17**, 254–266

Studies on Lysogeny in Staphylococci

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SUMMARY: A staphylococcus typing phage (NCTC 8426) was found to form lysogenic systems with three strains of staphylococci. In the case of two of these strains, staphylococci lysogenically infected with the typing phage were now lysed by the temperate phages they had previously carried. This loss of immunity was due to the replacement of the originally carried prophage by the prophage of phage 8426. This process could be reversed. With the third strain of staphylococcus the lysogenic offspring were not lysed by their original temperate phage, which they continued to carry. The NCTC phage 8426 appeared to undergo a modification when grown on the three strains described here, following which a few particles (*c.* 1 in 5×10^4) were able to lyse cells lysogenically infected with phage 8426. These virulent phage particles were maintained by propagation on lysogenic host cells but they reverted to the normal 8426 phage when grown on the non-lysogenic indicator. Finally phage 8426 showed another modification of its host range when grown on one of the three strains, this change was reversed when the modified phage was propagated on its normal host *Staphylococcus aureus* NCTC 8354.

During a recent study of ultraviolet irradiation of staphylococci (Gorrill & Gray, 1956) a number of staphylococci were lysogenically infected with typing phages. It was found that some combinations gave rise to lysogenic systems that were not completely immune to the carried phage. One such system has now been studied in more detail and the findings are recorded in this paper.

Staphylococcus-typing phage NCTC 8426 (phage 76, not now much used in typing, serological group F) is normally propagated upon *Staphylococcus aureus* NCTC 8354. When the phage and the sensitive staphylococcus are grown together it is possible to isolate colonies of staphylococci which appear to be lysogenically infected with phage 8426, and are quite stable on subculture. These staphylococci will be designated 8354/8426. When such staphylococci are tested for immunity to phage 8426 (grown on *S. aureus* 8354) they are found to show about one plaque for every 10^5 plaques formed on a fully sensitive indicator, e.g. staphylococcus 8354. When a plaque is picked from the lysogenic culture and grown in broth a phage is produced which gives about equal numbers of plaques when assayed on staphylococcus 8354 and staphylococcus 8354/8426. There exist, therefore, two forms of phage 8426. Occasionally cultures of staphylococci have been isolated which are lysogenically infected with phage 8426, but do not show this phenomenon, that is they appear to be totally resistant to both forms of phage 8426.

Further study of staphylococcus 8354/8426 showed that it is sensitive to phages to which the parent staphylococcus 8354 is resistant, the most striking example of this being the sensitivity of staphylococcus 8354/8426 to the temperate phage carried by the parent staphylococcus 8354, a phage which one would expect staphylococcus 8354/8426 itself to carry (Gorrill, 1956).

Phage 8426 is active against a number of strains of staphylococci; one other strain was found in which infection with phage 8426 rendered the bacteria sensitive to phages they previously carried. It is believed that these observations can be explained by the replacement of the normal prophage by prophage of phage 8426 during infection leading to lysogenization.

METHODS

The original medium, buffer and counting methods were described previously by Gorrill & Gray (1956). Unfortunately one of the constituents of the medium (Pronutrin, an enzymic digestion of casein, Herts Pharmaceuticals Ltd., Welwyn Garden City, Herts) ceased to be made during the course of these experiments. The remaining Pronutrin was reserved for broth making and a new source of agar medium sought. After prolonged trials batches of digest medium (tryptic digest of ox heart) which were completely satisfactory were obtained from the Southern Group Laboratory, Park Hospital, London, S.E. 13. Thirty ml. of medium was used in each 10 cm. Petri dish. The thickness of the layer of medium was found to influence the size and number of the plaques, thin plates gave very poor results even with otherwise satisfactory media. All plates were incubated at 30°, broth cultures at 37°.

Phage preparations were made by the methods described by Williams & Rippon (1952). Phage 8426 was made on staphylococcus 8354 except where otherwise stated. Where necessary phages were purified by two or three successive single plaque isolations. All phage preparations which had to be freed of bacteria were filtered through 'Gradocol' membranes, average pore size $0{\cdot}8\,\mu$ (Wright-Fleming Institute, St Mary's Hospital, London, W. 2).

Antisera were made by immunizing rabbits aged 6–9 months with high titre (10^9–10^{10}/ml.) phage stocks. The first injections were given subcutaneously, the later ones intravenously. The activity of the antisera was assayed by the methods described by Adams (1950). Phage counts were made either by spreading known dilutions over a lawn of sensitive staphylococci with a calibrated wire loop or by the soft-agar layer method.

Staphylococcus aureus strain NCTC 8354 was obtained from the National Collection of Type Cultures (NCTC), Colindale, London; strains 60, 98 and 105 from St Mary's Hospital, London, W. 2; all four strains were coagulase positive.

Typing phage 8426 was obtained from the National Collection of Type Cultures. Each of the four strains of staphylococci yielded a temperate phage; the four phages isolated will be named after the host staphylococci from which they were isolated, e.g. phage 8354 from staphylococcus 8354. There may be other temperate phages associated with these staphylococci, but it was not possible to obtain them in pure culture. When necessary cultures were induced by irradiation with ultraviolet light. The culture was suspended in buffer and irradiated at 35 cm. distance from a 45 W. ultraviolet lamp (Hanovia Ltd., Slough) for 60 sec. Two ml. samples were then added to 8 ml. broth and shaken at 37° C. until lysis had occurred.

Terminology. The staphylococcal typing phages and their propagating strains are identified by two different numbering systems, the laboratory numbers and the NCTC number (Williams & Rippon, 1952). In this paper the NCTC numbers will be used in conformity with the previous paper (Gorrill & Gray, 1956).

RESULTS

Interaction of staphylococcus 8354 *with phage* 8426

When phage 8426 was added to a broth culture of staphylococcus 8354 there was usually some initial clearing followed by an increase in turbidity so that after 24 hr. incubation the phage-treated culture was as dense as an untreated control. When such a culture was plated out, two different types of colony were seen, normal-looking colonies and colonies showing varying degrees of erosion and phage lysis.

The normal-looking colonies were found to be of two types, containing respectively normal non-infected staphylococci 8354 and staphylococci infected with phage 8426. Strains derived from colonies infected with phage 8426 were then examined to see if the presence of phage 8426 was due to the establishment of the lysogenic state or to phage contamination. If the culture was truly lysogenic then all the daughter colonies would show the presence of phage 8426 despite washing or treatment with antiphage serum. It was not found possible to rid these cultures of phage 8426, all daughter colonies contained the phage as was shown by replica-plating (Lederberg & Lederberg, 1952) on to sensitive indicator staphylococci. It was concluded that phage 8426 had established a lysogenic infection in staphylococcus 8354. In general a lysogenic strain is immune to its carried phage. When this test was carried out by spotting high-titre suspensions of phage 8426 on to the staphylococci lysogenic for phage 8426 it was found that about 1 particle in 10^5 of phage 8426 would form a plaque on the lysogenic staphylococci, and so it appeared that either immunity was not complete or phage 8426 existed in a second, highly virulent, form.

A number of isolations of such lysogenic staphylococci were made and in almost all cases they behaved as described above. In the records these staphylococci were recorded as 8354/8426/1–2–3, etc. as isolated. Finally it was decided that as all these cultures had the same properties it would be convenient to shorten the numbering and refer to them all as staphylococci 8354/6/1. It will be seen later that similar types of staphylococci can be isolated from different strains infected with phage 8426 and the expression 6/1 type was used to describe a staphylococcus lysogenically infected with phage 8426 but still sensitive to a small fraction (1 in 10^5) of phage 8426 particles.

As staphylococci are common laboratory contaminants two steps were taken to ensure that the lysogenic forms obtained were not contaminants. First, four separate cultures of staphylococcus 8354 and phage 8426 were used and clones of the 6/1 type could be regularly derived from them. Samples of such cultures were then freeze dried and at the end of the investigation a few were taken at random and sent to the Staphylococcal Reference Laboratory, Colindale, for phage-typing. It was found that the 6/1 strains were similar to one another and could have been derived from staphylococcus 8354.

During all the experiments with staphylococcus 8354 and phage 8426 three colonies were isolated which were lysogenically infected with phage 8426 and completely immune to high-titre suspensions of phage 8426. These were described in the records as A forms, they were phage typed and found to be similar to one another and different from the 6/1 strains. They will be discussed more fully later.

Table 1. *The effect of cross-spotting five strains of staphylococci with their sensitivity to* 8426 *phage for comparison*

Supernatants	Indicators				
	60	98	105	8354	8354/6/1
60	0	CL	0	0	CL
98	P±	0	P±	P±	P±
105	0	CL	0	0	CL
8354	SCL	SCL	SCL	0	P++ (faint)
8354/6/1	CL	CL (weak plaques)	CL	CL	0
8426 phage titre 10^{10}	10^{10}	10^{10}	10^{10}	10^{10}	4×10^5

CL = confluent lysis; SCL = semi-confluent lysis; P++, P+, P± = descending numbers of isolated plaques.

Change in phage sensitivity of staphylococcus 8354/6/1

A further interesting observation was made during a series of cross-spotting experiments, an example of which is given in Table 1. It will be seen that staphylococcus 8354/6/1 has now become sensitive to the phages in the supernatants of staphylococci 60 and 105 (phages 60 and 105). Staphylococcus 98 has been included as it is a good indicator for the phages employed (except phage 8426), and it will be seen that staphylococcus 8354/6/1 is comparable to staphylococcus 98 in its sensitivity to phages 60 and 105. Phage 8354 produces rather faint plaques on staphylococcus 8354/6/1, but they are comparable in numbers to these produced on staphylococcus 98. This increase in phage sensitivity was found in all strains of staphylococcus 8354/6/1 tested. A further point of interest is the difference in the quality of the plaques produced on staphylococcus 98 by the supernatants of staphylococci 8354 and 8354/6/1. The supernatant of the first gave rise to clear plaques characteristic of phage 8354 while the second gave only small ill-defined plaques similar to those given by phage 8426. It would seem from these results that staphylococcus 8354/6/1 had become sensitive to phages 60, 105 and 8354 and lost the ability to produce phage 8354, when compared with its parent culture staphylococcus 8354.

From these results it was concluded that when staphylococcus 8354 was exposed to phage 8426, the staphylococci lysogenic for phage 8426 which were obtained (6/1 types) had lost prophage 8354. This might be because introduction of the new prophage caused the old one to be lost; or it might be that a small proportion of the cells of strain 8354 spontaneously lost their prophage, and that only this minority of non-lysogenic cells could become lysogenic when exposed to phage 8426. A number of experiments were carried out to determine the number of cells of staphylococcus 8354 that were lysogenically infected with phage 8426, following multiple infection. $4{\cdot}5 \times 10^7$ cells/ml. of staphylococcus 8354 were exposed to $1{\cdot}2 \times 10^9$/ml. particles of 8426 phage at 37° C. for 30 min. The culture was then centrifuged and the supernatant counted for 8426 phage and 4×10^8/ml. particles remained; the multiplicity of infection therefore being about 19. The staphylococci were resuspended, diluted and aliquots spread on agar. The plates were then incubated and next day the number of colonies counted. These colonies were then replica printed against sensitive indicator media so that the number of phage-infected colonies could be scored. In this experiment 2×10^6/ml. staphylococci survived phage treatment and 50 % of these were phage infected. However, of these many were in the form of unstable or eroded colonies and in fact only 30 % of the phage-infected colonies were stable and behaved on further testing as 6/1 clones. Ignoring the unstable colonies for the moment, of the $4{\cdot}5 \times 10^7$/ml. staphylococci infected, 3×10^5/ml. formed 6/1 clones, i.e. slightly less than 1 %. These experiments were repeated several times and gave results of this order. Hence if staphylococcus 8354 is losing prophage 8354 it should be possible to show that rather under 1 % of its colonies are free of the phage. Cultures of staphylococcus 8354 were grown in broth and washed in buffer three times and then diluted and spread on digest plates and incubated to form well-developed isolated colonies. These colonies were then printed on to indicator staphylococci sensitive to phage 8354. In all, 1538 colonies were tested in this way and all contained phage 8354 and hence prophage 8354.

It seems likely that phage 8426 plays a part in the displacement of prophage 8354 and evidence of this was obtained by the study of the eroded colonies mentioned earlier. In all experiments where staphylococcus 8354 and phage 8426 were mixed eroded colonies were seen if the mixture was plated. In the experiment described above some 70 % of the colonies infected with phage 8426 were eroded. In a long series of experiments it was found that if a broth culture of an eroded colony was made, the culture after incubation always yielded a mixture of stable and eroded colonies. On examination the stable colonies were found to be either staphylococcus 8354 or 8354/6/1. The rates varying slightly from experiment to experiment. In one experiment eroded colonies were passed in daily subculture for 6 months. A possible explanation of this phenomenon is as follows: If a cell containing prophage 8354 is invaded by phage 8426 it might on subdivision give rise to cells containing only prophage 8354 or prophage 8426; that is, it would not replicate both prophages at the same time. If such a cell had been inoculated on to a plate the colony containing both staphylococcus 8354 and 8354/6/1 would be unstable and give rise to the appearance of eroded colonies. If grown in broth the liberation of free phage by the two lines of cells would also produce continual interaction and phage lysis. To test this theory an eroded colony was picked into broth and incubated at 37° C. overnight. It was then centrifuged three times to rid it of free phage and resuspended in buffer. Dilutions were made in broth and from these dilutions plates were spread with 0·1 ml. amounts and incubated. Suitable dilutions were examined under the microscope to ensure that the culture consisted of single, and pairs of, cocci and not clumps. The dilutions were now incubated at 37° and samples taken every hour for 6 hr. and 0·1 ml. amounts plated for colony counts. Next day it was possible to see which dilution originally contained about 100–400 cells/ml. yielding 10 to 40 colonies per plate. Following the fate of the bacteria at this dilution two observations were made. First the number of colonies declined for the first 3 hr. and the number of eroded colonies also declined. After 3 hr. the colonies increased in number and none were eroded. The plates were then printed on to plates layered with staphylococcus 8354 and the colonies were found to be either lysogenic (i.e. as measured against staphylococcus 8354) or not. Colonies of both types were picked off and examined and shown to be either staphylococcus 8354 (i.e. carrying phage 8354) or staphylococcus 8354/6/1. This experiment was repeated several times with similar results. It appears therefore that if cross-infection is avoided eroded colonies will finally yield a mixture of both strains of staphylococcus. As microscopy showed that the cells were in one's and two's, it seems that on incubation the cells either lyse or else divide into stable daughter cells containing only one prophage.

Phage in the supernatant of staphylococcus 8354 will form plaques on staphylococcus 8354/6/1. The titre of phage is low and it proved impossible to isolate cells of staphylococcus 8354/6/1 attacked by phage 8354. If phage 8354 was propagated on any of the sensitive staphylococci available it became contaminated by the lysogenic phages of the propagating strain. However, it was found that staphylococcus 8354 was inducible and following lysis phage titres as high as 5×10^{10}/ml. were obtained. Such a stock was filtered and tested for sterility and then spotted onto a lawn of staphylococcus 8354/6/1. From the bacterial growth in the centre of the spot an emulsion was prepared and freed of phage by centrifugation, and serial dilutions spread on plates. The majority of the colonies which grew were stable and all these proved to be staphylococcus 8354/6/1. However, there were a few eroded colonies and, following the results obtained with the earlier experiments on eroded colonies, cultures were set up. From these a mixture of stable and eroded colonies were formed, and on replica plating on staphylococcus 8354 it was found that the stable colonies were of two types, lysogenic and non-lysogenic. It was then shown that the stable 'non-lysogenic' colonies were staphylococcus 8354, i.e. they carried prophage 8354. It is therefore possible to turn staphylococcus 8354 into staphylococcus 8354/6/1 and back by phage action. Finally, some 1200 colonies of staphylococcus 8354/6/1 were printed against staphylococcus 8354 to ensure that the change was not due to the occurrence of a few cells that had spontaneously lost phage 8426. All the colonies tested were identical and contained 8426 phage.

Interaction of phage 8426 *with staphylococci* 60 *and* 105

These two staphylococci were part of a series of coagulase positive staphylococci isolated from patients in hospital. As will be seen from Table 1 their behaviour is very similar and their phage type is the same. Hence it may be assumed that they are two cultures of the same organism. Until the phage typing was done it was assumed they might be different and so all the experiments described below were done with both strains.

It is possible to isolate 6/1 strains easily following the addition of phage 8426 to the culture. On one occasion a strain lysogenically infected with phage 8426 was found which was resistant to both forms of phage 8426. This is the only A strain found in this group. As with staphylococcus 8354, the 6/1 strains were now sensitive to the phage they had previously carried, and it was not possible to show the presence of the original prophage in the new strains. It was possible to isolate eroded colonies which were similar in behaviour to these already described. Briefly then, these two strains of staphylococci behave in every way like staphylococcus 8354.

Interaction of phage 8426 *with staphylococcus* 98

This strain of staphylococcus was first included in these studies because it appeared to be a good indicator for 60, 105 and 8354 phage. It was known to give very small faint plaques with phage 8426 but in many ways this was an advantage as it allowed one of the first group to be counted in the presence of large amounts of phage 8426. When the action of phage 8426 on staphylococcus 98 was studied it was found that lysogenic strains could be produced and that they could be classified as 6/1 strains because they were sensitive to a few particles in stocks of phage 8426. Similarly, there was isolated one colony which failed to be lysed by the second form of phage 8426 and was classified as an 'A' strain.

There was, however, one important difference observed with the 98/6/1 strains, they were still immune to 98 phage and in fact 98 phage could be detected in their supernatant. In this case phage 8426 had not displaced the original prophage. Also while eroded colonies were seen in the early subcultures, they quickly disappeared on further subculture and yielded only 98/6/1 strains.

However staphylococcus 98 had one interesting property not shown by the other staphylococci described here, in that it seemed able to modify phage 8426 when it was propagated on it. If a lawn of staphylococcus 98 was inoculated with a dilution of phage 8426 so as to give discrete plaques it was noticed that a few were much clearer and bigger than the others. If such a plaque was picked out with a pasteur pipette into broth, shaken and used to inoculate a fresh lawn of staphylococcus 98 these plaques were all well defined. However, if the phage derived from a plaque of phage 8426 on staphylococcus 98 was counted on both staphylococcus 98 and staphylococcus 8354 it was found that it now gave a titre on staphylococcus 98 between 10 and 100 times higher than that on staphylococcus 8354. This difference was accentuated by several passages on staphylococcus 98 when the ratio would rise to 1000 to 1 or higher. If, however, a plaque that did appear on staphylococcus 8354 was taken it would contain phage which gave equal number of plaques on staphylococci 98 and 8354. This example of host modification of phage is less clear-cut than those seen with other phage systems but similar results have been found with other staphylococcal phages and will be discussed briefly later. Its main interest in this work was to render staphylococcus 98 an unsatisfactory strain for propagating phage 8426 or for testing single plaques of phage 8426.

'A' strains

There were in all five strains which were resistant to both types of 8426 phage while being lysogenically infected with phage 8426. Three were derived from staphylococcus 8354 and one each from staphylococci 105 and 98. When phage-typed they were lysed only by typing phages 53 or 53 and 75 and so were very similar. They were tested for the presence of the original prophage and like the 6/1 strains the 8354 and 105A strains had lost their original prophage while the 98A strain had not. When tested with phages 60, 98, 105 and 8354, all except 98A were sensitive to phage 98, none was sensitive to phage 8354 and the results with the other two were variable.

All five 'A' strains were tested with phages 8426, 8426v (described later) and phage 8354 and were shown to adsorb these phages as readily as the 6/1 strains, so their apparent immunity was not due to failure of the phage to adsorb. In one particular they were all unusual; when the supernatant of an overnight broth culture was examined it was found to contain about 10^7 phage particles/ml. which formed plaques on staphylococcus 8354 and was similar to phage 8426 in appearance. The supernatant however contained phage particles which would form plaques on the 6/1 strains, there were about 10^2–10^3 of these particles/ml. As will be seen later the supernatants of 6/1 strains do not contain particles which form plaques on 6/1 strains. It appears therefore that these A strains are different from the 6/1 strains in their phage sensitivity and in the production of a small number of altered forms of 8426 phage.

The two forms of 8426 *phage*

As was described earlier if high titre suspensions of phage 8426 are made on staphylococcus 8354 and then counted on lawns of staphylococci 8354 and 8354/6/1, it was found that a small fraction would form plaques on the 6/1 strains. If single plaques on a 6/1 lawn were taken they were found to contain phage which gave rather more plaques on staphylococcus 8354/6/1 than it did on staphylococcus 8354. This strain of phage 8426 which was capable of lysing cultures lysogenically infected with phage 8426 was designated phage 8426v (v = virulent). Phage 8426v would grow equally well on all 6/1 strains including those derived from staphylococci 60, 98 and 105.

Phage 8426v was made in bulk by propagation on lawns of staphylococcus 8354/6/1. It was found that if plaques formed by phage 8426v on staphylococcus 8354 were taken they contained very little phage 8426v and most of the phage particles were phage 8426. It appeared therefore that the change of phage 8426 to phage 8426v and back was determined in part at least by the strain of staphylococcus used for propagation. The reversal was also seen if staphylococci 60, 98 or 105 were substituted for staphylococcus 8354.

In order to study further the part played by the host cells in this change two groups of experiments were carried out. First, using the phage isolated from single plaques lawns of staphylococci were flooded and small volumes of high titre phage produced. These stocks of phage were then counted on lawns of staphylococci 8354 and 8354/6/1 which had been grown up from very small inocula (an overnight broth culture was diluted 10^{-6} in broth and then 1 ml. was added to 9 mls. for each stock and incubated). The counts were carried out on the same day with five cultures of each staphylococcus. Only a portion of the results is shown in Table 2. It will be seen that with high titre stocks of phage 8426 about 1 in 5×10^4 particles form plaques on staphylococcus 8354/6/1. With phage 8426v there were commonly two to four times as many plaques formed on staphylococcus 8354/6/1 as on staphylococcus 8354. These results were not influenced by the use of different subcultures of the two staphylococci.

Table 2. *Stocks of phages* 8426 *and* 8426*v counted on indicators staphylococci* 8354 *and* 8354/6/1, *figures show plaque forming particles per ml.*

Stocks of phage 8426	Cultures of staphylococcus 8354			Cultures of staphylococcus 8354/6/1		
	1	2	3	1	2	3
1	2×10^{9}	$1{\cdot}2 \times 10^{9}$	$2{\cdot}4 \times 10^{9}$	$4{\cdot}2 \times 10^{4}$	$5{\cdot}1 \times 10^{4}$	$3{\cdot}3 \times 10^{4}$
2	2×10^{9}	$2{\cdot}1 \times 10^{9}$	$2{\cdot}6 \times 10^{9}$	$3{\cdot}3 \times 10^{4}$	$6{\cdot}1 \times 10^{4}$	$8{\cdot}7 \times 10^{4}$
3	9×10^{9}	6×10^{9}	8×10^{9}	$1{\cdot}5 \times 10^{5}$	2×10^{5}	6×10^{4}
4	2×10^{9}	$2{\cdot}8 \times 10^{9}$	$2{\cdot}9 \times 10^{9}$	4×10^{4}	$2{\cdot}2 \times 10^{4}$	$3{\cdot}1 \times 10^{4}$
5	3×10^{10}	$2{\cdot}7 \times 10^{10}$	$1{\cdot}9 \times 10^{10}$	$2{\cdot}5 \times 10^{5}$	$4{\cdot}1 \times 10^{5}$	$4{\cdot}9 \times 10^{5}$
Stocks of phage 8426v						
1	$5{\cdot}5 \times 10^{7}$	$6{\cdot}0 \times 10^{7}$	$3{\cdot}3 \times 10^{7}$	$4{\cdot}1 \times 10^{7}$	$8{\cdot}9 \times 10^{7}$	$4{\cdot}7 \times 10^{7}$
2	$1{\cdot}4 \times 10^{7}$	$1{\cdot}1 \times 10^{7}$	$1{\cdot}2 \times 10^{7}$	$5{\cdot}1 \times 10^{7}$	$6{\cdot}8 \times 10^{7}$	$5{\cdot}7 \times 10^{7}$
3	$1{\cdot}1 \times 10^{10}$	$1{\cdot}2 \times 10^{10}$	$9{\cdot}8 \times 10^{9}$	$2{\cdot}1 \times 10^{10}$	$2{\cdot}9 \times 10^{10}$	$1{\cdot}9 \times 10^{10}$
4	$5{\cdot}9 \times 10^{7}$	$7{\cdot}5 \times 10^{7}$	$6{\cdot}5 \times 10^{7}$	$1{\cdot}1 \times 10^{8}$	$2{\cdot}1 \times 10^{8}$	$7{\cdot}7 \times 10^{8}$
5	$1{\cdot}3 \times 10^{9}$	$1{\cdot}9 \times 10^{9}$	$1{\cdot}5 \times 10^{9}$	$1{\cdot}6 \times 10^{9}$	$3{\cdot}3 \times 10^{9}$	$9{\cdot}7 \times 10^{8}$

In the other experiments single plaques were taken with a pasteur pipette, emulsified in buffer (10 ml.) and the number of phage particles counted on several subcultures of the two indicator staphylococci as described above. The results are illustrated in Table 3 which shows that the change from phage 8426v to phage 8426 was related to the host cell (staphylococcus 8354 or 8354/6/1) on which it was grown. Further experiments were done in which aged cultures and very young shaken cultures were used and in no case was a consistent trend seen which would allow one to say that the bacterial cultures varied significantly in the degree to which they brought about this change.

Finally, attempts were made to produce one-step growth curves but as is common in this group of phages (Rountree, 1955) infection of sensitive cells in broth is not always followed by a burst. However, on two occasions it was found that when staphylococcus 8354 was infected with a low (0·1) multiplicity of phage 8426 v and diluted suitably a burst followed at about 90 min. with the liberation of an average of 18 phage particles per infected cell. These particles were all phage 8426.

Table 3. *Results of counting the phage content of various plaques on different cultures of the two indicators*

A = plaques of 8426 phage from lawns of staphylococcus 8354; B = plaques of 8426 phage from lawns of staphylococcus 8354/6/1; C = plaques of 8426 v phage from lawns of staphylococcus 8354; D = plaques of 8426 v phage from lawns of staphylococcus 8354/6/1.

	Cultures of staphylococcus 8354			Cultures of staphylococcus 8354/6/1		
Plaque	1	2	3	1	2	3
A1	7×10^6	$2{\cdot}5\times10^6$	5×10^6	7×10^2	2×10^2	4×10^2
A2	$2{\cdot}5\times10^7$	$1{\cdot}6\times10^7$	$2{\cdot}0\times10^7$	$1{\cdot}1\times10^3$	$3{\cdot}5\times10^3$	$1{\cdot}2\times10^3$
A3	$6{\cdot}1\times10^6$	$6{\cdot}3\times10^6$	$4{\cdot}1\times10^6$	3×10^2	3×10^2	4×10^2
B1	$7{\cdot}6\times10^5$	$8{\cdot}1\times10^5$	$4{\cdot}9\times10^5$	$2{\cdot}1\times10^6$	$1{\cdot}5\times10^6$	$2{\cdot}4\times10^6$
B2	$2{\cdot}2\times10^5$	4×10^4	3×10^5	$1{\cdot}9\times10^5$	$2{\cdot}4\times10^5$	$4{\cdot}0\times10^5$
B3	$4{\cdot}8\times10^4$	$2{\cdot}1\times10^5$	$8{\cdot}9\times10^4$	$3{\cdot}0\times10^5$	$8{\cdot}8\times10^4$	$3{\cdot}0\times10^5$
C1	$6{\cdot}0\times10^6$	$7{\cdot}2\times10^6$	$4{\cdot}1\times10^6$	2×10^2	3×10^2	8×10^2
C2	$4{\cdot}5\times10^6$	$5{\cdot}1\times10^6$	$8{\cdot}0\times10^6$	9×10^2	0*	8×10^2
C3	$1{\cdot}1\times10^7$	$8{\cdot}0\times10^6$	$2{\cdot}5\times10^6$	7×10^2	6×10^2	2×10^2
D1	$2{\cdot}9\times10^5$	$3{\cdot}5\times10^5$	$8{\cdot}0\times10^4$	$4{\cdot}4\times10^5$	$3{\cdot}1\times10^5$	$6{\cdot}6\times10^5$
D2	$4{\cdot}4\times10^5$	$6{\cdot}1\times10^5$	$2{\cdot}0\times10^5$	$6{\cdot}2\times10^5$	$8{\cdot}1\times10^5$	9×10^5
D3	$6{\cdot}7\times10^5$	$2{\cdot}1\times10^5$	$4{\cdot}1\times10^5$	$1{\cdot}6\times10^6$	$8{\cdot}4\times10^5$	$2{\cdot}0\times10^6$

* Less than 100 phage particles/ml.

All attempts to isolate staphylococci lysogenically infected with phage 8426 v were unsuccessful. It was thought that it might play some part in the formation of the 'A' strains which do liberate a small proportion of their phage as 8426 v, unlike the 6/1 strains as described below.

At this point it is convenient to review the evidence that phage 8426 v is in fact modified phage 8426. Briefly, on indicators such as staphylococci 98, 8354 and 105 their plaques are similar, they are inactivated at the same rate by antisera and are equally heat sensitive. They adsorb at the same rate on to both staphylococcus 8354 and 8354/6/1.

Normally phages propagated on staphylococcus 8354 while contaminated with phage 8354 would appear pure on lysogenic offspring of staphylococcus 8354 as these cultures are usually immune to the temperate phages of the parent. However in this case, owing to the removal of prophage 8354 in the making of the staphylococcus 8354/6/1 strains, phage 8354 could and in fact did multiply in staphylococcus 8354/6/1. It was shown that phage 8426 v was not an adapted form of phage 8354 by the fact that they were serologically different and if plaques of phage 8354 grown on staphylococcus 8354/6/1 were taken and assayed on staphylococcus 98, plaques of phage 8354 were seen, together with the small plaques of phage 8426 derived from the surrounding bacteria. There was no evidence therefore that phage 8354 present in phage 8426 as a contaminant in any way adapted during growth on staphylococcus 8354/6/1 and was mistaken for a virulent form of phage 8426.

If the stocks of phage 8426 made on staphylococcus 8354 were examined it was seen that the virulent variant was present in all of them in the ratio of 1 in 10^5 or more. Now if broth cultures of staphylococcus 8354/6/1 were centrifuged the supernatant was found to contain about 10^7 8426 phage particles/ml. but no 8426v phage. Similarly, if cultures of staphylococcus 8354/6/1 are induced large quantities of phage 8426 are released ($>10^{10}$/ml.), but in fifteen tests no 8426v phage was found. The possibility that phage 8426v was the result of a recombination between phages 8354 and 8426 was considered. It was rejected on the grounds that strains of phage 8426v could be made on staphylococcus 60 and 105 and less well on staphylococcus 98 and these strains of phage 8426v appeared identical. On the other hand, the prophages in these staphylococci were certainly different. Finally, unlike the 6/1 strains, the 5A strains did seem to produce spontaneously a small proportion of phage 8426v.

DISCUSSION

There are two examples of host modification of phage 8426 in this paper. Staphylococcus 98 produces a variant which forms plaques to a much lower titre on staphylococcus 8354 than on staphylococcus 98, while staphylococcus 8354/6/1 appears to produce rare variants which are virulent.

The ability of some strains of bacteria to modify the phage propagated on them has been known for some time and was reviewed by Luria (1953). Very briefly, if a phage is grown for only one cycle in these modifying strains, it is changed and can now only rarely form plaques on its original indicator. However, these rare plaques contain normal phage and it can be shown that the return to normality follows a single cycle of growth. The ability of a few phage particles modified by the bacteria to grow on the original host is due to the presence in the original host of a few cells capable of supporting the growth of the phenotypically modified phage particles. The number of these cells varies with the physiological state of the culture and is also modified by irradiation.

In an extensive study of the typing phages of *Salmonella typhi* Anderson and his co-workers (1953*a*, *b*, 1955, 1956) have shown that adaptation of a single phage to form a series of specific typing phages involves in some cases phenotypic modification, in others selection of host range mutants and in still others both processes. In general cultures which were lysogenically infected with unrelated 'O' phages selected host-range mutants while the non-lysogenic cells selected phenotypic variants.

In the cases reported here the results are less clear-cut. Staphylococcus 98 modifies phage 8426 but the effect is strengthened by repeated passage. This has been seen with other staphylococcal phages (Rountree, 1956). Further study of the modifying effect of staphylococcus 98 was not made.

Considerably greater study was devoted to the change of phage 8426 to phage 8426v. It was essential first to ensure that the appearance of phage '8426v' was not due in fact to a contaminant phage picked up during propagation of phage 8426. The most likely phage would have been phage 8354 but it is fairly certain that this possibility may be excluded. Also by complex crossing of phages from one indicator to another it seemed possible to show that the change of phage 8426 to 8426 v was due to a modification of phage 8426 by the propagating staphylococci and not to picking up a contaminant phage. As described earlier the change occurred during growth on a sensitive propagating strain. Owing to the difficulties of producing single growth cycles it was not possible to analyse the change in detail, but it was possible in two cases to show the change of phage 8426 v to phage 8426 in one-step growth curves. Since it is known that the number of cells capable of supporting the growth of phenotypically modified phage varies with the physiological state of the culture, it was expected that the ratio of phage 8426 to 8426 v in different stocks would vary on different cultures of the same staphylococcus. Such was not the case, although this negative evidence would not justify one in rejecting the hypothesis of phenotypic change. On the other hand, the reversal of phage 8426 v to 8426 when grown on staphylococcus 8354 was so constant a feature as to exclude the possibility that the change resulted from mutation. For it has been shown that if a culture selects a mutant phage then if that mutant is now propagated on the original host it retains its character. Phage 8426 v was thought to be a virulent phage (Jacob *et al.* 1953) in that it never formed a lysogenic system and it lysed cells containing the prophage of its temperate variant (Jacob & Wollman, 1953).

The behaviour of the A strains in producing small quantities of virulent phage 8426 spontaneously was interesting, but it was by no means certain in what other fashion they differed from the corresponding 6/1 strains. It was therefore impossible to use these observations to suggest the mechanism by which phage 8426 was modified. It seems most likely that it is an example of phenotypic variation where the variant is virulent, but it would be unwise to be dogmatic in such a technically different field.

Bertani (1954) studied the problem of the interaction of prophage and invading phage. In order to ensure interaction he infected cells with mutants of a carried prophage and was able to show that substitution or double infection could follow. Boyd (1956) has suggested that the immunity a prophage confers on a cell with regard to another phage may vary from complete to one where the incoming phage displaces the original prophage, usually taking its place but sometimes not, resulting in a 'curing' of the phage infection. In the system studied here it is possible to displace either prophage if an excess of the other is present. It is interesting that the cells may contain both phages for a time but the situation is unstable and always results in daughter cells containing only one prophage. Stable double lysogenics did not occur, nor did 'curing' as far as could be seen. Presumably phage 8426, when reduced to prophage, occupies a similar position on the chromosome to prophages 60, 105 and 8354 but different from that occupied by prophage 98.

REFERENCES

Adams, M. H. (1950). Methods of study of bacterial viruses. *Meth. med. Res.* **2**, 1.

Anderson, E. S. & Felix, A. (1953*a*). 'Degraded Vi strains' and variation in Vi phage II of *Salmonella typhi*. *J. gen. Microbiol.* **8**, 408.

Anderson, E. S. & Felix, A. (1953*b*). The Vi type-determining phages carried by *Salmonella typhi*. *J. gen. Microbiol.* **9**, 65.

Anderson, E. S. & Frazer, A. (1955). The influence of the factors determining Vi-type specificity in *Salmonella typhi* on the adaptation of Vi Phage II. *J. gen. Microbiol.* **13**, 519.

Anderson, E. S. & Frazer, A. (1956). The statistical distribution of phenotypically modifiable particles and host-range mutants in populations of Vi-phage II. *J. gen. Microbiol.* **15**, 225.

Bertani, G. (1954). Studies on lysogenesis. III. Superinfection of lysogenic *Shigella dysenteriae* with temperate mutants of the carried phage. *J. Bact.* **67**, 696.

Boyd, J. S. K. (1956). Immunity of lysogenic bacteria. *Nature, Lond.* **178**, 141.

Gorrill, R. H. (1956). Effect of superinfection on phage sensitivity in staphylococci. *Nature, Lond.* **178**, 91.

Gorrill, R. H. & Gray, R. A. (1956). The induction of bacteriophage in staphylococci. *J. gen. Microbiol.* **14**, 167.

Jacob, F. & Wollman, E. L. (1953). Induction of phage development in lysogenic bacteria. *Cold Spr. Harb. Symp. quant. Biol.* **18**, 101.

Jacob, F., Lwoff, A., Siminovitch, A. & Wollman, E. L. (1953). Définition de quelques termes relatifs à la lysogénie. *Ann. Inst. Pasteur*, **84**, 222.

Lederberg, J. & Lederberg, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63**, 399.

Luria, S. E. (1953). Host-induced modification of viruses. *Cold Spr. Harb. Symp. quant. Biol.* **18**, 237.

Rountree, B. M. (1955). The role of divalent cations in the multiplications of staphylococcal bacteriophages. *J. gen. Microbiol.* **12**, 275.

Rountree, P. M. (1956). Variations in a related series of staphylococcal bacteriophages. *J. gen. Microbiol.* **15**, 266.

Williams, R. E. O. & Rippon, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg., Camb.* **50**, 320.

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Hošt'álek, Z. (1957). *J. gen. Microbiol.* **17**, 267–273

The Maintenance of *Eremothecium ashbyi*

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SUMMARY: Different methods of maintenance of highly flavinogenic strains of *Eremothecium ashbyi* have been studied.

Storage for 6 months at room temperature or 4° on agar slants either untreated, layered with liquid paraffin or sealed with paraffin wax resulted in considerable loss of flavinogenic capacity. Similar losses also occurred when various strains were transferred regularly at 2-week intervals.

Complete maintenance of flavinogenic activity over 8 months was obtained by freeze-drying cultures, previously frozen at −68°, in maltose or glucose solutions in either water or serum.

In 1935 Guilliermond described *Eremothecium ashbyi*, the parasite of cotton-bolls; on more detailed study this parasite was found to produce a yellow pigment of flavin nature (Guilliermond, Fontaine & Raffy, 1935), which was later identified spectrographically as riboflavin (Raffy, 1938).

The basic study of the physiology of this organism was made by Schopfer (1944). Other authors (e.g. MacLaren, 1952; Dikanskaya, 1953; Hickey, 1953; Goodwin & Pendlington, 1954) followed the nutritional requirements of *Eremothecium ashbyi* and the mechanism of riboflavin synthesis. Recently the results of the older studies are being verified by radioisotopic methods using compounds labelled with ^{14}C (e.g. McNutt, 1954; Klungsøyr, 1954).

Eremothecium ashbyi was maintained by most authors by passage once a month (Hickey, 1953) or once a week (MacLaren, 1952) on agar slants of similar composition as the liquid media used. Moore & de Becze (1947) consider this the most reliable method of maintaining this organism. By varying the composition of the slants the activity of the strain did not diminish.

Opinions about the maintenance of vitality and biosynthetic activity of *Eremothecium ashbyi*, cultivated on solid media without transfer, vary. Hickey (1953) states that his strain maintained viability on a glucosomaltose agar with yeast extract for 2–3 months. Moore & de Becze (1947) maintained their strain at room temperature for 6 months without loss of biosynthetic activity. When kept in a refrigerator, the strain suffered a considerable loss of riboflavin productivity. Dikanskaya (1954), on the other hand, kept *E. ashbyi* both on solid and on liquid media in the refrigerator for several months, without noting any loss of productivity.

Very little is known about freeze-drying of *Eremothecium ashbyi*. Moore & de Becze (1947) found that *E. ashbyi* on freeze-drying loses its ability to produce riboflavin. In most cases this change was irreversible. Hickey (1953) also states that freeze-drying of *E. ashbyi* was not successful. None of these authors described the method of freeze-drying used, nor did they record results in detail.

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METHODS

Organism. Eremothecium ashbyi originated from the collection of the Department of General Microbiology, Charles University. From a heterogeneous strain with an average riboflavin production of about 170 μg./ml., 150 isolates were obtained. Monospore isolates were prepared by cutting out the germinating spore by means of a special pneumatic isolator (Nečásek, Palečková & Tesař, 1953). For the selection of isolates with maximum riboflavin production a screening test was used based on the fluorescence in ultraviolet light of the zones surrounding the colonies (Rytíř, 1955). Three isolates with greatest riboflavin production were used for further experiments.

Cultivation. A liquid medium of the following composition was used: glucose, 1 % (w/v); peptone, 1 % (w/v); yeast autolysate (49 % dry substance), 0·2 % (w/v); distilled water; pH 6·8 before sterilization; sterilized for 20 min. at 1·5 atm. The solid medium contained, in addition to the above ingredients, 1·5 % (w/v) agar.

The organism was cultivated in 500 ml. flasks containing 50 ml. of medium on a reciprocating shaking machine (96 swings/min., amplitude 9·5 cm.) at 28° ± 1°.

A standard inoculum was used throughout. It consisted of 0·5 ml. of a 2-day shake culture previously inoculated with a spore suspension (2 ml.) from a 2- or 3-day agar slant. Three-day slants were used when freeze-dried material was examined.

Riboflavin determinations were carried out on 7-day cultures. Differences between three parallel flasks were not more than ± 5 % of the mean for the three flasks.

Storage on agar slants. The following methods of conservation were used:

(1) *Agar slopes.*

(2) *Agar slopes closed by paraffin wax*—the stopper of the inoculated agar slant was cut off level with the top of the tube and the end of the test tube was then immersed several times in molten paraffin wax.

(3) *Agar slopes, layered by liquid paraffin*—the test tube was slanted so that the end of agar reached approximately to the centre of the test tube; after inoculation it was layered by sterile paraffin oil (of a quality required by Czechoslovak Pharmacopoea) to a level of about 2 cm. above the top of agar.

The cultures were stored either at room temperature, or at 4°. All agar slants were incubated for 6 days at 28° prior to conservation.

Freeze-drying. The method utilizing a system of two test tubes was used (Šourek, 1955). The storage test tubes (7 × 40 mm.) were inserted into thick-walled test tubes (9 × 180 mm.) which after desiccation were closed by fusing under vacuum. Agar cultures were used for freeze-drying. The basal solid medium contained 3 % (w/v) agar, in order to prevent contamination of the freeze-dried space by the cultivation-medium during transfer of the culture. The sterilized conservation test tubes were filled by two drops of the freeze-drying medium; the mycelium was well mixed with the inoculating loop by rubbing against the wall of the test tube and then washed down to the freeze-drying medium. Prior to drying, the material was either frozen for 30 min. in a mixture of glycerol, acetone and solid CO_2, or kept for 30 min. at 4°. It was then dried twice, 4 hr. each, with a 16 hr. long interruption, over P_2O_5 at 0·5 mm. Hg. (During the interruption in drying the material was kept in an evacuated desiccator over P_2O_5.) The freeze-dried samples were stored in darkness at room temperature. For inoculation freeze-dried cultures were shaken up with the basal liquid medium and transferred to an agar slant as a mash. The first control inoculation was made immediately after drying.

Determination of riboflavin. 5 ml. medium were diluted with 40 ml. distilled water, acidified with dilute HCl to pH 4·5 and hydrolysed for 1 hr. at 1 atm. After hydrolysis the sample was made up to 50 ml. and filtered.

Riboflavin in the filtrate was determined polarographically by the method of standard addition (Heyrovský & Zuman, 1953). The determination was carried out in an acetate buffer (Walpole), pH 5·4 in a Heyrovský polarographic cell flushed with nitrogen. Cathodic polarographic curves were recorded from zero potential against the potential of the pool of mercury in the cell. High sensitivity of the galvanometer was used and compensation of the capacity current was therefore applied. E.m.f. 3·8 V. (200 mV./abscissa). The concentration of riboflavin was calculated from the difference between the values of the limiting currents (height of the waves) before and after the addition of a standard solution, according to the formula given in the literature cited above.

RESULTS

Storage on agar slopes

Three monospore isolates (average production: A, 327 μg./ml.; B, 325 μg./ml.; C, 312 μg./ml.) were stored on agar slopes, on agar slopes closed by paraffin wax and on agar slopes layered with liquid paraffin, and kept either at room-temperature or at 4°. They were examined after 2, 4, 6 and 8 months of storage.

Table 1 shows that the flavinogenic capacity of all the stored cultures decreased at approximately the same rate irrespective of the method of storage. After 2 months the decrease was slight but after 8 months the riboflavin levels were the same as those of the heterogeneous strain from which these isolates were originally obtained.

Freeze-drying

The following freeze-drying media were tested: distilled water, saline, dried milk (Difco), whey, 5 % (w/v) and 10 % (w/v) solutions of glucose, 5 % (w/v) and 10 % (w/v) solutions of glucose and maltose in serum. Cultures were dried either unfrozen or after freezing at −68°. Two-day non-sporulating cultures and 6-day richly sporulating cultures were stored.

Table 2 shows that the best method of storing freeze-dried *Eremothecium ashbyi* is to lyophilize actively sporulating cultures after first freezing them at −68°, using a 5 or 10 % (w/v) glucose solution in water or a 5 or 10 % solution of glucose or maltose in serum. Under these conditions the flavinogenic activity was not significantly reduced after 8 months storage. Distilled water, saline and serum are particularly unsuitable as freeze-drying media.

Table 1. *Production of riboflavin by strongly flavinogenic isolates of* Eremothecium ashbyi *after storage on agar slants*

Seven-day shake cultures (50 ml. medium in 500 ml. flask) triplicate analysis; temp. 28°; riboflavin in μg./ml.

Storage time (months)	Isolate	Method of storage					
		Agar slant Storage temp.		Agar slant closed by paraffin wax Storage temp.		Agar slant covered by liquid paraffin Storage temp.	
		Room temperature	4°	Room temperature	4°	Room temperature	4°
2	A	325	290	329	308	326	319
	B	304	306	327	322	304	346
	C	304	283	337	342	355	277
4	A	240	245	249	263	247	241
	B	246	286	230	243	253	235
	C	228	264	252	226	273	238
6	A	233	216	217	247	206	241
	B	244	215	215	216	213	226
	C	236	223	216	229	232	218
8	A	*	151	184	207	200	205
	B	*	136	194	200	202	196
	C	*	157	210	225	191	188

Original riboflavin levels produced by these isolates were: A, 327 μg./ml.; B, 325 μg./ml.; C, 312 μg./ml.

* The culture did not grow.

Cultures which had been stored for 5 months on agar under paraffin wax, i.e. heterogeneous material with low production were also freeze-dried under optimal conditions. Storage experiments with this material showed a progressive decline in flavinogenic power (Table 3).

Maintenance of strains by regular transfer

The three cultures used in the freeze-drying experiments were also transferred regularly every 2 weeks, in order to compare this with other methods of storage. Riboflavin production was determined at 2, 4 and 6-monthly intervals. It is evident from Table 4, that the flavinogenic capacity of the cultures decreases after twelve transfers to that of the heterogeneous strain.

DISCUSSION

As discussed in the Introduction the general opinion in the literature is that serial transfer of most strains of *Eremothecium ashbyi* at 1- to 3-monthly intervals maintains their riboflavin producing power intact. The present work (Table 4) indicates that using strongly flavinogenic strains this view was not

Table 2. *Riboflavin production by a strongly flavinogenic isolate of* Eremothecium ashbyi, *after being freeze-dried under different conditions*

Experimental conditions as in Table 1. Riboflavin in $\mu g./ml.$

		Method of freeze-drying										
		Distil. water	Saline	Dried milk	Whey	Serum	5 % glucose	10 % glucose	5 % glucose in serum	10 % glucose in serum	5 % maltose in serum	10 % maltose in serum
Unsporulated unfrozen culture	Immediately after freeze-drying	205	*	246	239	*	266	248	313	303	284	301
	After 8 months storage	149	*	182	*	*	*	193	219	227	247	214
Unsporulated frozen culture	Immediately after freeze-drying	*	*	289	*	*	307	*	*	*	325	305
	After 8 months storage	*	*	*	156	*	214	*	*	*	254	221
Sporulated unfrozen culture	Immediately after freeze-drying	187	*	201	268	134	248	*	253	204	289	263
	After 8 months storage	*	*	*	223	*	209	231	185	170	201	246
Sporulated frozen culture	Immediately after freeze-drying	*	*	275	303	*	397	321	343	394	465	410
	After 8 months storage	*	*	253	265	*	284	298	348	332	368	348

Riboflavin production prior to freeze-drying 327 $\mu g./ml.$

* The cultures did not grow.

Table 3. *Production of riboflavin by a poorly flavinogenic strain of* Eremothecium ashbyi, *after being freeze-dried under various conditions*

Experimental conditions as in Table 1. Riboflavin in μg./ml.

		Method of freeze-drying			
Time of storage	Culture	5% maltose	5% glucose	5% maltose in serum	5% glucose in serum
Immediately after freeze-drying	A	218	206	181	188
	B	212	193	200	205
	C	210	198	207	195
3 months	A	176	182	184	183
	B	185	188	192	195
	C	179	179	174	175
6 months	A	190	181	173	180
	B	181	191	173	166
	C	181	169	180	171

Riboflavin production prior to freeze-drying: culture A, 217 μg./ml.; B, 215 μg./ml.; C, 219 μg./ml.

Table 4. *Production of riboflavin (μg./ml.) by poorly flavinogenic isolates of* Eremothecium ashbyi *maintained by regular subculture*

The organisms were subcultured every 2 weeks. Cultures were incubated at 28° for 6 days and then stored at room temperature. Experimental details as Table 1.

Time of subculture (months)	Riboflavin μg./ml.		
	Isolate A	Isolate B	Isolate C
0	217	215	219
2	206	212	198
4	199	189	199
6	167	171	170

upheld. Furthermore, agar slants sealed in various ways also begin to lose their flavinogenic activity after 2 months storage, either at room temperature or at 4° (Table 1).

Previous authors (Moore & de Becze, 1947; Hickey, 1953) have dismissed freeze-drying as unsuitable for conserving *Eremothecium ashbyi* but they did not report in detail the methods used. Conditions have now been defined which allow production of freeze-dried cultures which completely retain their flavinogenic activity over a period of at least 8 months. Any variations in these conditions result in loss of activity.

The author wishes to express his gratitude to Docent Dr J. Stárka for his valuable advice and for his interest in this work. He further wishes to express his thanks to Dr J. Šourek (Institute of Epidemiology and Microbiology, Prague) for consultation in the freeze-drying methods and for kind permission for use of the freeze-drying apparatus.

REFERENCES

Dikanskaya, E. M. (1953). The study of nutritional physiology by *Eremothecium ashbyi.* (Issledovaniya po fiziologii pitaniya *Eremothecium ashbyi.*) *Microbiology, Moscow*, **22**, 256.

Dikanskaya, E. M. (1954). The production of riboflavin by microfungi *Eremothecium ashbyii.* (Obrazovanie riboflavina mikroskopitsheskim gribom *Eremothecium ashbyii.*) *Trud. Inst. Mikrobiol.* (USSR), **3**, 35.

Goodwin, T. W. & Pendlington, S. (1954). Riboflavin in *Eremothecium ashbyii.* Nitrogen metabolism and flavinogenesis in *Eremothecium ashbyii. Biochem. J.* **57**, 631.

Guilliermond, A. (1935). Sur un champignon nouveau, parasite des capsules du cotonnier, *l'Eremothecium ashbyii* et ses relations possibles avec le *Spermophthora gossypii* et les Ascomycetes. *C.R. Acad. Sci., Paris*, **200**, 1556.

Guilliermond, A., Fontaine, M. & Raffy, A. (1935). Sur l'existence dans *l'Eremothecium ashybii* d'un pigment jaune, se rapportant au groupe des flavines. *C.R. Acad. Sci., Paris*, **201**, 1077.

Heyrovský, J. & Zuman, P. (1953). *An Introduction in Practical Polarography.* (Úvod do praktické polarografie.) 2nd ed. Prague: Czechoslovak Academy of Sciences.

Hickey, R. J. (1953). Some nutritional requirements for biosynthesis of riboflavin by *Eremothecium ashbyii. J. Bact.* **66**, 27.

Klungsøyr, L. (1954). The biosynthesis of riboflavin in *Eremothecium ashbyii. Acta chem. scand.* **8**, 723.

MacLaren, J. A. (1952). The effect of certain purines and pyrimidines upon the production of riboflavin by *Eremothecium ashbyii. J. Bact.* **63**, 233.

McNutt, W. S. (1954). The direct contribution of adenine to the biogenesis of riboflavin by *Eremothecium ashbyii. J. biol. Chem.* **210**, 511.

Moore, H. N. & de Becze, G. (1947). Riboflavin synthesis by *Eremothecium ashbyii* in synthetic and natural substrates. *J. Bact.* **54**, 40.

Nečásek, J., Palečková, F. & Tesař, A. (1953). A mechanism for monosporic and monocellular isolation. (Zařízení pro monosporickou a jednobuněčnou isolaci.) *Čsl. pat.* 84,005.

Raffy, A. (1938). Spectrographie de fluorescence de la flavine *d'Eremothecium ashbyii* en solution et des cultures de ce champignon. *C.R. Soc. Biol., Paris*, **128**, 392.

Rytíř, V. (1955). *Selection and evaluation of productive strain Eremothecium ashbyii.* (*Výběr a hodnocení produkčního kmene Eremothecium ashbyii.*) Thesis, Charles University, Prague.

Schopfer, W. H. (1944). La biotine, l'aneurine et le mésoinositol, facteurs de croissance pour *Eremothecium ashbyii* Guilliermond. *Helv. chim. acta*, **27**, 1017.

Šourek, J. (1955). Preservation of pathogenic microorganisms by freeze-drying. (Konservace pathogenních mikroorganismů lyofilisací.) *Česk. hyg. epid. mikrobiol. imunol.* **4**, 329.

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WILLIAMS, M. A. & RITTENBERG, S. C. (1957). *J. gen. Microbiol.* **17**, 274–278

Nuclear Fusion in *Spirillum* spp.

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SUMMARY: Cellular fusion of entwined fresh-water spirilla, *Spirillum sinuosum* and *S. anulus*, is followed by nuclear fusion. Evidence of nuclear fusion was obtained from impression smears (fixed in acetic-alcohol, hydrolysed in N-HCl and stained with Giemsa) made at regular time intervals during the growth of cultures of these organisms. Following cellular fusion and nuclear fusion of opposing granules into rings of six granules, with the coalescence of the rings into a single, elongate body, the fusion cells enlarge into 'giant' organisms. The chromatinic bodies of these giants divide or separate into numerous rod-like chromatin structures around the vacuoles found in the later stages. The giant organisms either give off or fragment into small rod-like bodies, containing, at first, a triangular-shaped chromatinic structure. This triangular chromatinic structure condenses into a single spherical body as the small rod-like organisms are more clearly formed. The fate of these small rod-like organisms is unknown, but similar rod-like bodies which develop into the normal spirillum have been observed in young cultures of both *S. sinuosum* and *S. anulus*. Nothing resembling classical mitosis or meiosis was observed in any of the stained preparations of either organism.

Cellular fusion has been shown to occur in the marine organism, *Spirillum lunatum* (Williams & Rittenberg, 1956). In this organism, cellular fusion occurs after the spirilla have become entwined and results in the production of microcysts. The phenomenon of entwined spirilla is common to all the spirilla species examined and, by analogy with the process in *S. lunatum*, it might be assumed that cellular fusion also occurs in the other species. To determine whether this hypothesis is correct and whether nuclear fusion follows cellular fusion, two fresh-water species, *S. sinuosum* and *S. anulus*, were selected for study. *S. sinuosum* was chosen because more entwined spirilla occur in cultures of this species than in all others we have studied, and *S. anulus* was selected because of its large size.

Robinow (1956) has objected to calling the chromatin bodies found in bacteria *nuclei* on the basis that the term 'nucleus' is defined as 'something that arises from a set of chromosomes' and that, as yet, chromosomes have not been demonstrated in the chromatin bodies of bacteria. The term 'nucleus' however, is in common usage in bacterial literature to indicate the chromatin bodies of bacteria with the implicit understanding that the use of the term in this connexion does not necessarily imply that these bodies are the exact equivalent of the nucleus of higher organisms; nor that typical mitosis and meiosis such as occur in higher organisms has been established in bacteria. It is in this sense that the term 'nucleus' is used throughout this paper.

METHODS

Nutrient broth of the following composition was used for the cultivation of both experimental organisms: soybean peptone (Case Laboratories, Chicago, Illinois), 0·5 g.; beef extract, 0·3 g.; yeast autolysate (Basamin, Anheuser-Busch Inc., St Louis, Missouri), 0·3 g.; sea water, 100 ml.; distilled water, 900 ml. For solid media, 15 g. agar/l. were added to the liquid medium. Growth occurred very slowly in broth cultures of both *Spirillum sinuosum* and *S. anulus* when the routine bacteriological method of using one loopful of material for transfer was followed. Under these conditions, as a rule, there was no visible turbidity until 48–72 hr. after transfer. For this reason one drop of a broth culture was always used as the inoculum.

Aliquots of broth cultures were examined by dark-phase contrast, in wet mount preparations, during the course of development of the cultures to determine when entwined organisms were most abundant, thus establishing the proper times for making nuclear-stained preparations. For the latter purpose, using 7-day broth cultures as inocula, series of broth cultures of both organisms were prepared. These were incubated at 30° for periods of 12, 16, 20, 24, 30, 48 and 60 hr., at which times the tubes were centrifuged and the sedimented organisms then spread over the surface of nutrient agar plates. These plates were incubated at 30° for 2 hr., after which time small agar squares were removed, aseptically, at 2 hr. intervals and impression smears made on glass slides. The preparations were fixed in a solution of 1 part of glacial acetic acid, 3 parts of absolute alcohol for 5 min., passed through a graded series of ethanol and distilled water dilution to distilled water and hydrolysed in N-HCl solution at 60° for 8 min. After hydrolysis the smears were rinsed in distilled water and stained with Giemsa solution in phosphate buffer (pH 7·0) for 30 min.

The smears were examined with the Leitz Ortholux microscope, equipped with an achromatic condenser, an apochromatic objective (×90) N.A. 1·25, and compensating oculars (×12). The photographs were made with the Leitz Aristophot camera using Panatomic X sheet film and a 560 mμ. interference filter.

RESULTS

The number of entwined organisms found in a broth culture of either *Spirillum sinuosum* or *S. anulus* is very small in comparison with the number of those not entwined. As a rule, entwined cells are most numerous in routine broth cultures 24–36 hr. after inoculation, although entwined cells may be found as early as 18 hr. and as late as 5 days after inoculation.

Spirillum sinuosum grows as long slightly curved filaments although shorter more spiral forms are always present in every culture. In the entwining process of this organism, the filamentous spirillum bends near the centre and one end winds around the other from the centre down (Pl. 1, figs. 1A, B), usually leaving the terminal ends free. In *S. anulus*, which usually occurs as an S-shaped organism, entwining takes place in daughter organisms after division but before separation (Pl. 2, figs. 3A, B). In both organisms fusion follows entwinement (Pl. 1, fig. 1B; Pl. 2, fig. 3B).

Stained preparations show that fusion of the chromatinic granules occurs after fusion in both species. Different aspects of the process are shown in Pl. 1, fig. 1. Fig. 1A shows a spirillum prior to fusion in which the chromatinic granules are aligned opposite each other. Although it is difficult to determine the exact number of these granules at a particular focal depth, there appear to be three granules for each spiral curve of the organism. The other photographs in Pl. 1, fig. 1, are of fused spirilla after fusion of the chromatinic granules. Two appearances predominate: one is the formation of rings from the 'six' opposing granules by fusion of adjacent granules (Pl. 1, figs. 1B, C). The second is the fusion of single granules in the opposing spiral curves (Pl. 1, fig. 1D). It is possible that the two different appearances are merely due to the angle from which they are viewed and that fusion occurs in the same manner in all the organisms.

Beyond this stage a multitude of patterns of the chromatinic material are found and since serial observations cannot be made on a single organism it is difficult, or impossible to construct a sequence of events. As far as can be ascertained from the examination of a large number of preparations, all the granules in opposing spirals of fused spirilla eventually coalesce into a single, intensely staining, elongate body, which can be seen in Pl. 1, figs. 1E, F.

After fusion of the chromatinic granules occurs or just prior to this event, the fused spirilla enlarge to about twice their normal diameter, forming what we have termed 'giant' forms. These are shown in Pl. 1, fig. 2. Giant forms are very difficult to find in the impression smears and several preparations may be examined before finding them. They stain very poorly and are much less resistant to hydrolysis than the ordinary spirillum. Based on the frequency of the normal, the entwined, and the giant forms at various time intervals during the development of a culture, it is our impression that all fused organisms of *Spirillum sinuosum* and *S. anulus* enlarge into giant forms, although the evidence is certainly not conclusive. Unfused ends of the original spirillum are clearly seen at the bottom of the organisms shown in Pl. 1, fig. 2A, and less clearly in the organism in Pl. 1, fig. 2C. Enlargement into giant forms occurs only after fusion, but appears independent of the stage of fusion of the chromatinic granules, as can be observed by an examination of Pl. 1, fig. 2.

Further changes occur in the chromatinic bodies of the giant forms of *Spirillum sinuosum* following which the organisms separate into individual, somewhat angular bodies. Similar changes occur in *S. anulus*. Because of the larger size and better staining characteristics of the latter species, it is used to illustrate the later stages. Pl. 2, fig. 3A, shows an entwined spirillum of *S. anulus* and a normal sized spirillum. Pl. 2, fig. 3B, shows an entwined spirillum after fusion, with the chromatin fusion already initiated. Pl. 2, figs. 3C–3H show the enlargement of the fused spirillum into giant forms 3 to 4 times the size of the normal organism. In the sequence shown the chromatin fuses into single bodies (Pl. 2, figs. 3B–3D). These bodies undergo rearrangement and reorient themselves into a horizontal bar-like structure which divides longitudinally into smaller bar-like structures (Pl. 2, fig. 3D). These in turn separate into still smaller structures (Pl. 2, fig. 3E), the principal feature of which appears to be a bar with a granule at each end. This type of configuration is more clearly observed in Pl. 2, figs. 3F and 3G (marked with an arrow). It has not been possible to reach a decision as to the mechanism by which this separation of the chromatinic bodies occurs, although separation clearly does occur, as can be observed in Pl. 2, fig. 3H.

In giant forms, occurring during the later stages of growth of the organism in broth cultures (60–72 hr.), the sequence of events occurring in the chromatinic bodies can be interpreted. The giant forms found in the early stages of growth in broth cultures (36–48 hr. after inoculation) continue to enlarge in size until they are approximately 3 to 4 times the size of the ordinary spirillum. At this time, the chromatinic bodies appear in the shape of horizontal rod-like bodies (Pl. 2, fig. 4A) and the vacuoles begin to appear between (or in) these rod-like bodies (Pl. 2, figs. 4B, C); these rod-like bodies then separate into two smaller rods around the edge of the vacuoles (Pl. 2, figs. 4D, E). An examination of Pl. 2, figs. 4D and E, will show that there appears to be a granule at each end of these rod-like structures (marked with an arrow). The giant forms, at this stage, either fragment or give off small rod-like bodies (Pl. 2, fig. 4F). Pl. 2, fig. 4G, shows these rod-like bodies as they are coming off the giant forms. The chromatin in the small rods at first appears to consist of a triangular segment which condenses into a single spherical body as the cells are more clearly formed. The fate of these small rod-like forms is unknown, although the presence of very similar small rods (Pl. 2, fig. 4H) has been noted in young broth cultures of both *Spirillum sinuosum* and *S. anulus*. It is possible that these small rod-like forms represent the growth of the small rod-like bodies observed in Pl. 2, fig. 4G, and that they develop into the small spirilla shown in Pl. 2, fig. 4H-3 and H-4.

Although the data indicate that the chromatinic granules of the paired spirilla fuse into single bodies and these in turn separate into smaller units, the mechanism and significance of both the fusion process and the separation process are still unknown. In none of the photographs presented is there any evidence of configurations resembling those found during meiosis or mitosis in higher organisms.

We should like to express our appreciation to Dr C. C. Lindegren, Southern Illinois University, Carbondale, Illinois, for the use of his laboratory facilities during the course of this study. The material presented in this paper forms a part of the thesis presented by one of us (M.A.W.) to the faculty of the University of Southern California, in partial fulfilment of the requirements for the Ph.D. degree.

REFERENCES

Robinow, C. F. (1956). The chromatin bodies of bacteria. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 181.

Williams, M. A. & Rittenberg, S. C. (1956). Microcyst formation and germination in *Spirillum lunatum*. *J. gen. Microbiol.* **15**, 205.

EXPLANATION OF PLATES

Spirillum sinuosum and *S. anulus* were both grown in broth and the spirilla were transferred to agar, as cited in text. All photographs are of hydrolysed and stained organisms. Times shown in parentheses include growth from the time of inoculation until preparations were made. Arrows indicate configurations discussed in text. Magnification, ×1750 (Pl. 1); ×2333·33 (Pl. 2).

Plate 1. *Spirillum sinuosum*

Fig. 1A. Entwined spirillum prior to fusion, showing chromatinic granules aligned opposite each other (23 hr.).

Fig. 1B. Fusion form showing formation of 'rings' from the 'six' opposing granules by fusion of adjacent granules (24 hr.).

Fig. 1C. Fusion form in which 'rings' of granules are fusing together (26 hr.).

Fig. 1D. Fusion form in which single chromatin granules, in opposing spiral curves, are fusing (22 hr.).

Figs. 1E, F. Fusion forms in which 'rings' have fused into an intensely staining, elongate body (29 and 30 hr. respectively).

Figs. 2A, B. Fusion forms enlarging into 'giant' forms (34 and 36 hr. respectively).

Fig. 2C. Giant form showing fusion of chromatinic granules (36 hr.).

Fig. 2D. Giant form in which chromatinic granules have completely fused (40 hr.).

Plate 2. *Spirillum anulus*

Fig. 3A. Fusion of two daughter forms (18 hr.).

Fig. 3B. Fusion of chromatinic granules in fused forms (24 hr.).

Fig. 3C. Giant forms in which fused nuclei are seen as horizontal bar-like structures (28 hr.).

Figs. 3D, E. Giant forms showing beginning division or separation of the horizontal bar-like structures into two smaller bars (30 and 34 hr., respectively).

Figs. 3F, G. Giant forms showing bar-like chromatinic structures with granules at each end (39 and 42 hr., respectively).

Fig. 3H. Giant form showing small bar-like chromatinic structures with a granule at each end, separating around the vacuoles into still smaller structures (48 hr.).

Fig. 4A. Giant forms, showing bar-like chromatinic structures. (50 hr.).

Fig. 4B. Giant form, showing separation of bar-like chromatinic structures around vacuoles with granules at each end (50 hr.).

Figs. 4C–E. Giant forms, showing separation of bar-like chromatinic structures around vacuoles (52, 52 and 58 hr., respectively).

Fig. 4F. Giant form, in which triangular body has either separated or broken off the end (66 hr.).

Fig. 4G. Triangular and spherical shaped bodies originating from giant forms, showing the intensely staining triangular and spherical chromatinic bodies (92 hr.).

Fig. 4H. 7-day broth culture transferred to broth and incubated for 12 hr. before being plated out on agar. 4H-1. Small rod-like bodies (14 hr.). 4H-2. Small form morphologically recognizable as a spirillum (16 hr.). 4H-3. Small spirilla (18 hr.). 4H-4. Small spirillum (24 hr.).

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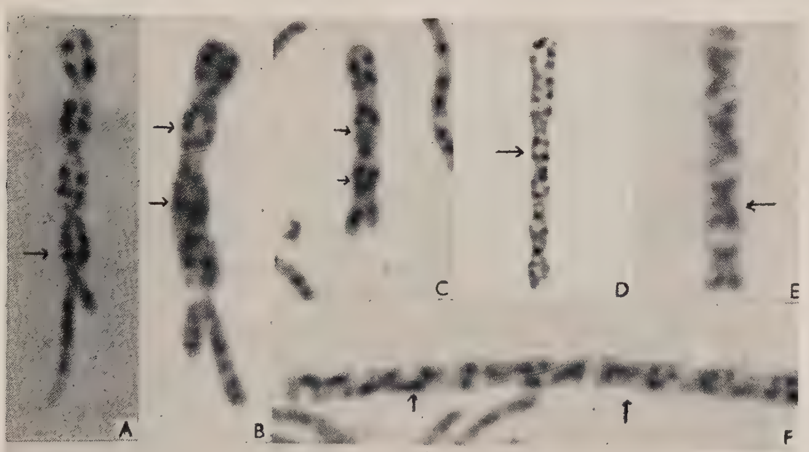

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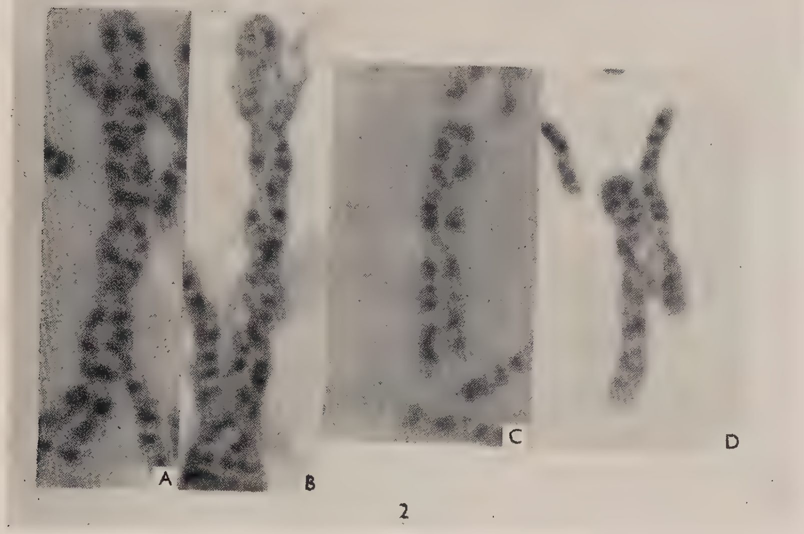

2

M. A. WILLIAMS & S. C. RITTENBERG—NUCLEAR FUSION IN *SPIRILLUM* SPP. PLATE 1

(*Facing p.* 278)

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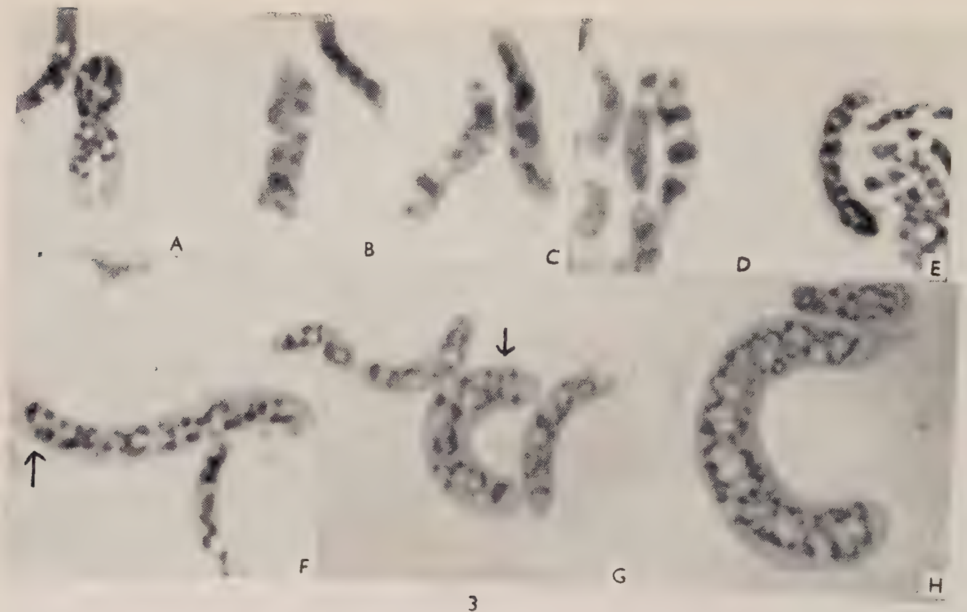

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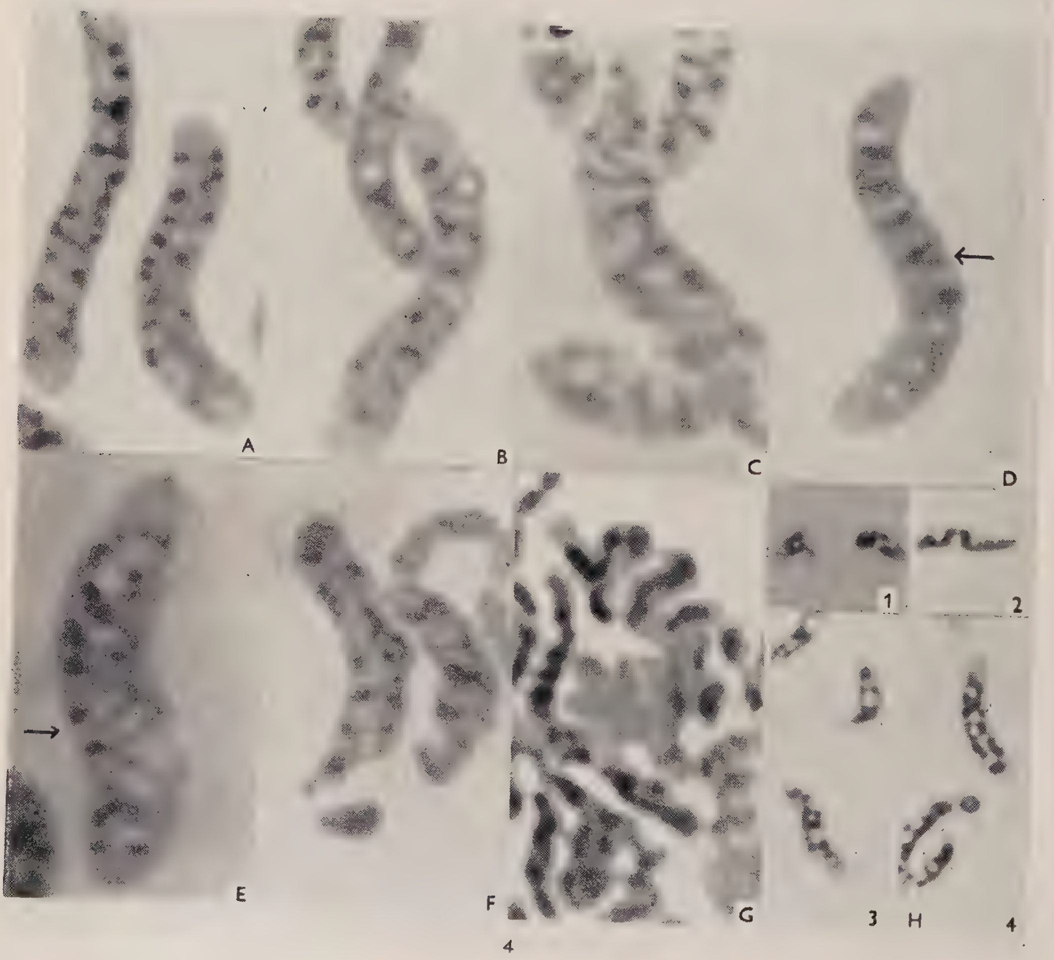
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4

M. A. Williams & S. C. Rittenberg—Nuclear fusion in *Spirillum* spp. Plate 2

ROWATT, E. (1957). *J. gen. Microbiol.* 17, 279–296

Some Factors Affecting the Growth of *Bordetella pertussis*

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SUMMARY: *Bordetella pertussis* incubated in liquid media is prevented from multiplying by autoclaved cysteine and is rapidly killed by another substance, possibly a peroxide. The inhibition by autoclaved cysteine is overcome by serum albumin and the inhibition by 'peroxide' is overcome by red blood corpuscles, haemin, or ferrous sulphate sterilized by filtration. In a medium containing casein hydrolysate and yeast extract, logarithmic growth may continue for 40–50 hr. at one rate and then may change to a slower rate which continues for a further 20–30 hr. before growth stops. The initial growth rate can be maintained throughout by adding high concentrations of whole blood. The yield of organisms is proportional to the concentration of glutamic acid up to 0·3 % (w/v). It is possible that growth at greater concentrations is stopped by the high pH value caused by the oxidation of glutamate. Omission of casein hydrolysate decreases the yield of bacteria. A larger yield is obtained by the use of a large inoculum of bacteria with adherent blood.

Until 1939, *Bordetella pertussis* could not be grown in liquid media unless blood or tissue extracts were added. In that year Hornibrook (1939) obtained growth in a casein hydrolysate medium containing starch and cysteine. Small inocula would not grow in this medium, and in the modified medium introduced by Cohen & Wheeler (1946) the organism did not grow unless the initial inoculum was more than 10^6 bacteria/ml. of medium. Cultures have been grown from 1000 or fewer organisms/ml. in casein hydrolysate previously treated with charcoal and fat solvents (Fisher, 1948) or with charcoal at different pH values (Jebb & Tomlinson, 1955). Proom (1955) showed that colloidal sulphur and sulphides and heated cysteine are important inhibitors of the growth of *B. pertussis* and when steps are taken to exclude these compounds small inocula will grow in Cohen & Wheeler's medium (see Rowatt, 1955). Hornibrook (1940), Proom (1955) and Jebb & Tomlinson (1955) all describe fairly simple defined media for the growth of *B. pertussis*. All contain nicotinamide, glutamic acid and cysteine or cystine, but differ in the other components. These media giving slow, rather limited growth are inadequate for growing large quantities of bacteria for vaccines or metabolic studies.

To obtain good growth of bacteria three main conditions must be observed: growth must begin promptly; the logarithmic growth rate must be rapid and must be maintained throughout the growth period; and sufficient nutrients must be present to give a high final yield of viable organisms. Some of the substances and conditions necessary to give good growth of *Bordetella pertussis* are detailed below.

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METHODS

Organisms. Virulent strains of *Bordetella pertussis* were obtained from public health laboratories and dried by the method of Greaves (1944) at the third subculture after isolation on Bordet-Gengou medium. The strain M2400, which was less easily adapted to blood agar than other strains, was used throughout the investigation; for the experiments reported here it was dried at the second subculture after mouse passage. These strains were maintained on Bordet-Gengou (BG) medium W.H.O. report, 1953) and discarded at the fourteenth (48 hr.) subculture when another dried culture was opened. 'Pertussis A', obtained from Dr Pollock, was stored dried at the eighth subculture from the original isolation (see Pollock, 1947) and maintained on blood agar (BA, 5% (v/v) defibrinated horse or human blood). The rest of the strains were maintained on digest agar plates (DA, a tryptic digest of horse meat) and dried cultures were opened when required. The strains of *B. parapertussis* and *B. bronchiseptica* were obtained from the National Collection of Type Cultures (NCTC). The phase IV strains of *B. pertussis* were: CN2217, a strain derived from the phase I CN1262 by the Wellcome Research Laboratories; NCTC 364, the original phase IV strain of Leslie & Gardner (1931) acquired by the NCTC in 1920; G154E and G146E obtained from Glaxo Laboratories Ltd. G154E contained a heat-stable antigen agglutinated by serum against the heat-stable antigen of phase I *B. pertussis*. All cultures were incubated at 35° C.

Small-scale growth experiments

Inocula for small-scale experiments were grown on BG plates for 24–48 hr. and suspended in phosphate buffered saline (pH 7·2), diluted 1/1000, and 0·02 ml. was added to 20 ml. medium (total dilution 10^{-6}). Expressed as numbers of viable bacteria/ml. medium, the inocula were between 10^2 and 10^4 equivalent to 10^{-6}–10^{-8} mg. dry wt./ml.

Apparatus. Media (20 ml.) in 100 ml. Pyrex Erlenmeyer flasks capped with 30 ml. beakers were incubated at 35° on a horizontal shaker (Kantorowicz, 1951). The same shelf of the shaker was always used. Glassware was cleaned in ethanolic potash followed by concentrated HCl with $NaNO_3$ (flasks, test tubes and beakers) or by 2·5 N-HCl (pipettes) and rinsed with glass-distilled water. Pipettes were plugged with cotton wool.

Media used contained the same ingredients as Cohen & Wheeler's (1946) medium except in the details listed below:

	Nitrogen source		Method of sterilization		
Medium	Casamino Acids (% w/v)	Glutamic acid (% w/v)	Yeast extract	Cysteine	$FeSO_4$
CWa	1	0	Seitz-filtration	Autoclaving	Autoclaving
CW1	1	0	Seitz-filtration	Seitz-filtration	Autoclaving
CW2	Varied	0·14	Seitz-filtration	Seitz-filtration	Autoclaving
CW3	0·1	0·14	Seitz-filtration	Seitz-filtration	Autoclaving
CW4	0·1	0·14	Seitz-filtration	Seitz-filtration	Seitz-filtration

Yeast extract was an aqueous extract of fresh yeast (see Rowatt, 1955). Casamino Acids, unless otherwise specified, was Bacto-vitamin-free Casamino Acids (Difco). It was autoclaved in 10% (w/v) solution at 120° for 20 min. before being autoclaved with the rest of the medium. $MgCl_2$ was replaced by the equivalent quantity of $MgSO_4.7H_2O$ (0·48 mg./ml.).

Media were autoclaved in bulk and then dispensed into flasks. Amino acids were obtained from Roche Products Ltd. One sample of haemin was obtained from this firm, another was kindly supplied by Messrs L. Light and Co. Ltd. A recrystallized sample was given by Professor C. Rimington. Red blood cells were centrifuged from human blood, washed with saline and the packed cells lysed with distilled water. They were added aseptically to the autoclaved media. Oleic acid was purified from a commercial sample by low temperature crystallization (Smith, 1939).

Large-scale growth experiments

Inocula for large-scale experiments were grown on BG slopes in 6 × 1 in. test tubes for 24 hr. Approximately 3 ml. 1% (w/v) Casamino Acids (pH 7) were added to all of the tubes and then the bacteria were emulsified with a Pasteur pipette and the suspensions pooled and diluted such that 5 ml. could be added to each 500 ml. medium. Five to nine tubes were used for 5 l. medium, giving a concentration of approximately 10^7 viable organisms/ml. medium.

Apparatus. Media (500 ml. unless otherwise stated) were autoclaved in penicillin flasks plugged with cotton wool and incubated as above. The most rapid rate of shaking was used except where otherwise stated. Glassware was cleaned with detergent in the routine washing up room and was not strictly grease-free.

Media were made as Cohen & Wheeler's (1946) medium except that $MgSO_4$ and yeast extract were used as above and Casamino Acids was replaced by a pancreatic hydrolysate of casein obtained from Benger Laboratories Ltd., Holmes Chapel. Cysteine and yeast extract were autoclaved with the rest of the medium unless otherwise stated. The media were:

	Nitrogen source		Method of sterilization		
Medium	Casein hydrolysate (% w/v)	Glutamic acid (% w/v)	Yeast extract	Cysteine	Concn. of cysteine (mg./ml.)
LS1	1	0·1	Autoclaving	Autoclaving	0·03
LS2	1	Varied	Autoclaving	Autoclaving	0·03
LS3	0	0·5	Autoclaving	Autoclaving	0·03
LS4	0	0·5	Autoclaving	Seitz-filtration	Varied

Estimations

Concentration of organisms. Viable counts were made by the method of Miles & Misra (1938) on BG medium using 1% (w/v) Casamino Acids as diluent. One flask was removed from the incubator for each sample. Concentration of suspensions of organisms was measured by a Gambrell photoelectric colorimeter and expressed as mg. dry weight/ml. (see Rowatt, 1955).

Iron estimation. Iron was estimated with $\alpha\alpha'$-dipyridyl by the modification of Thorp (1941).

Haemin estimation. Haemin was estimated by the method of Rimington (1942).

Other estimations were carried out as described previously (Rowatt, 1955).

RESULTS

Effect of various conditions on the lag period

Condition of the inoculum. In the experiments reported here the inoculum was adjusted to give a final concentration of 10^2–10^3 viable organisms/ml. in the final growth medium. In the standard CW1 medium containing filtered cysteine, the lag phase lasted 5–17 hr. It was measured by finding the point of intersection of the logarithmic phase and a line drawn at the level of the initial inoculum. The differences in length of lag phase were partly due to age of inoculum because, although some 48 hr. inocula gave a lag period of less than 12 hr., none of the 24 hr. inocula showed lag periods of more than 12 hr. In this medium, the addition of lysed red blood cells shortened the lag phase in all experiments: in that shown in Fig. 1 the length of the lag period was halved.

Effect of autoclaved cysteine. Proom (1955) showed that colloidal sulphur and sulphides which are formed when cysteine is autoclaved inhibited the growth of *Bordetella pertussis.* Although Cohen & Wheeler (1946) obtained growth of *B. pertussis* from large inocula in the presence of autoclaved cysteine, with a small inoculum (10^3) the bacteria did not multiply and began to die after about 50 hr. (Fig. 1). It is not known whether increase of cell substance was inhibited or only the actual division of the bacteria, because only the number of viable bacteria was measured at this stage. The inhibition was overcome by adding 1/1000 whole blood or lysed red blood cells; higher dilutions of these materials gave growth more slowly. Fisher (1948) showed that crude serum albumin allowed growth of *B. pertussis* from small inocula in autoclaved media, and serum or electrophoretically pure serum albumin replaced red blood cells in my experiments. However, growth with serum (1/400) was slower than that with the concentration of lysed red blood cells (1/1000) containing the equivalent concentration of protein per unit volume. The course of the growth curve with serum was not found but it is possible that albumin was as efficient as red blood cells in overcoming colloidal sulphide and sulphur inhibition but unable to maintain the primary growth rate (see p. 287).

Cysteine sterilized by filtration overcomes inhibition by colloidal sulphur (Woiwod, 1954). Colloidal sulphur may be present in casein hydrolysates but the amount in Casamino Acids was small because omission of cysteine from CW1 leaving the yeast extract as the sole source of sulphydryl groups did not slow growth significantly. However, Jebb & Tomlinson (1955) grew *Bordetella pertussis* from small inocula in media autoclaved with cystine in which the Casamino Acids had been treated with activated charcoal. Using autoclaved cysteine instead of cystine, I obtained growth when the Casamino Acids had been treated with one batch of activated charcoal (British Drug Houses Ltd.) but not with another batch of the same make or with a batch of Norite. Autoclaved cystine was as inhibitory as autoclaved cysteine. Consequently it seems probable either that a compound increasing the formation of inhibitor from cysteine or cystine was removed by the charcoal, or that one overcoming the inhibition was added.

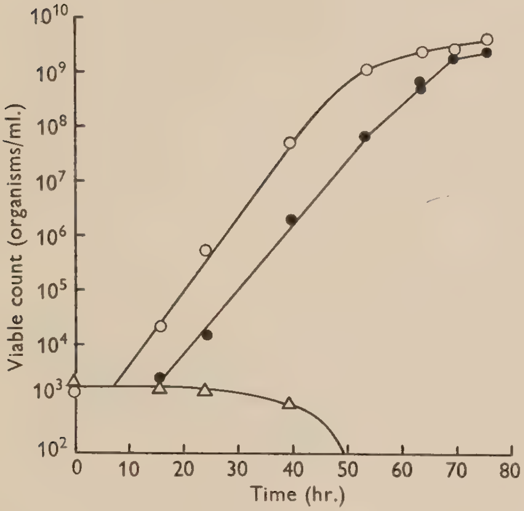

Fig. 1

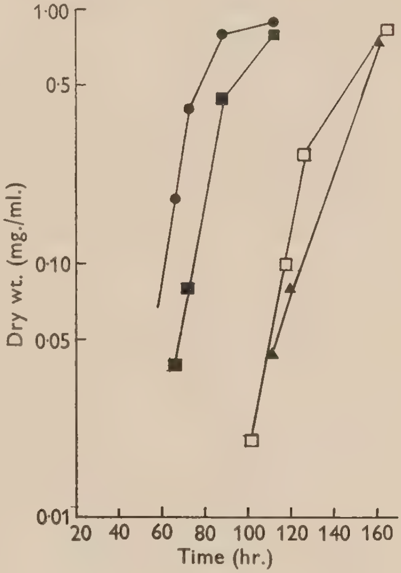

Fig. 2

Fig. 1. Inhibition of growth of *Bordetella pertussis* by autoclaved cysteine. Cultures incubated at 35° in 20 ml. medium. The medium contained 1 % (w/v) Casamino Acids with cysteine autoclaved in the medium (CWa) or sterilized by Seitz-filtration (CW1) or CW1+1/100 lysed R.B.C. Strain 467. CWa, △—△; CW1, ●—●; CW1+blood, ○—○.

Fig. 2. Effect of concentration of Casamino Acids on growth of *Bordetella pertussis*. Cultures incubated at 35° in 20 ml. medium CW2 containing 0·14 % (w/v) glutamic acid and stated concentration of vitamin-free Casamino Acids (*B. pertussis* strain 467) or Casamino Acids (iron-free) (*B. pertussis* strain 593). Inocula *c.* 10^3 bacteria/ml. Casamino Acids vitamin-free: 1 %, ●—●; 0·7 %, ■—■; 0·5 %, ▲—▲; Casamino Acids iron-free: 0·7 %, ○—○.

Effects of albumin. Pollock (1947) found that a degraded strain of *Bordetella pertussis*, 'Pertussis A', would not grow in broth unless albumin was added, and oleic acid (4 μg./ml.) prevented the growth so obtained. At this concentration of oleic acid, growth of phase I *B. pertussis* in CW1 was not affected. At 5 times the concentration (25 μg./ml.) visible growth was prevented, but 1/1000 lysed red blood cells did not affect the inhibition. The inhibition by oleic acid, therefore, although it was also overcome by albumin seemed due to a different effect from that by autoclaved cysteine.

Effect of low concentrations of Casamino Acids. In the experiments quoted above, the medium contained 1 % (w/v) Difco Bacto-vitamin-free Casamino Acids. When the concentration of Casamino Acids was decreased and glutamic acid added instead (see below), the period before a visible turbidity appeared became longer: in 1 % (w/v) Casamino Acids a given turbidity was reached 10 hr. earlier than in 0·7 % (w/v) Casamino Acids and 60 hr. earlier than in 0·5 % (w/v) Casamino Acids (Fig. 2); 0·3 % (w/v) Casamino Acids in this medium (CW 2) did not give visible turbidity. Viable counts showed that with the lower concentrations of Casamino Acids the lag period was longer although the growth rate was unaffected. For example, in an experiment with 1 % (w/v) Casamino Acids the M.G.T. was 2·7 and the lag period about 10 hr.; with 0·4 % the M.G.T. was 2·5 and the lag period 40 hr. Bacteria incubated in media containing 0·1 % (w/v) Casamino Acids (CW 3) died rapidly (Fig. 3); the colony count had fallen considerably after 18 hr. In some experiments in which the organisms grew after a long lag period it seemed that only a small proportion of the original inoculum grew. The colony count fell abruptly and all bacteria seemed to be dead; as soon as sufficient bacteria were present to be measured, they were growing at the normal logarithmic rate. This was quite different from the effect with autoclaved cysteine when the viable count remained the same for nearly 40 hr. (Fig. 1).

Effect of red blood cells. The addition of whole blood allowed growth in 0·1 % (w/v) Casamino Acids (CW 3) giving a lag period of the same length as or shorter than that in 1 % (w/v) Casamino Acids (Fig. 3). This time the activity was in the red cells. In the experiment shown in Table 1, growth with 1/4000 plasma was much slower than that with 1/10,000 lysed red blood cells and 1/40,000 plasma did not give growth at all, although 1/100,000 red blood cells gave growth as rapidly as 1/10,000 red blood cells. As the red blood cells contain 2·5 times as much protein per unit volume as plasma does, the effect cannot be due to protein as such.

Table 1. *Growth of* Bordetella pertussis *in 0·1 % Casamino Acids with* R.B.C. *and plasma*

Cultures incubated at 35° in 20 ml. CW 3 medium (0·1 % w/v Casamino Acids) with stated concentrations of R.B.C. and plasma. Strain M 2400, inoculum approx. 10^3 bacteria/ml.

	Dry wt. (mg./ml.)				
		CW 3 plus			
		R.B.C.		Plasma	
Time of incubation (hr.)	CW 3 alone	1/10,000	1/100,000	1/4,000	1/40,000
56	—	0·040	0·035	—	—
115	—	—	—	0·150	—
288	<0·01	—	—	—	<0·01

The quantity of red blood cells required varied with different batches of media. In some experiments, 1/500,000 red blood cells gave as rapid growth as 1/10,000 and in others 1/50,000 gave slightly slower growth than 1/10,000 red blood cells. The same variation was found with CW 2 containing 0·4 % (w/v) Casamino Acids which on one occasion had a lag period of only 24 hr. compared with 40 hr. in the experiment quoted above.

Effect of haemin. The high activity of blood in promoting growth in CW3 suggested that haemin might be responsible. When this compound was added to the medium before autoclaving the organisms grew, but they generally grew more slowly than with lysed red blood cells with a lengthened lag period; with 2·5 µg. haemin/ml., the lag period was usually about 25 hr. although in one experiment it was 65 hr. The M.G.T. was still the same as with 1/10,000 red blood cells (Fig. 3).

Haemin which had been sterilized by filtration through a Gradocol membrane gave a visible turbidity more rapidly than the same quantity of haemin sterilized by autoclaving (Table 2). In most experiments, 0·25 µg. haemin /ml. was not enough to promote growth, but in one experiment growth with this concentration of filtered haemin and also with autoclaved haemin (2·5 µg. /ml.) was as rapid as with 1/10,000 lysed red blood cells. This suggests that the variation is related to the medium rather than the haemin. Recrystallized haemin gave as variable growth as the commercial samples.

Table 2. *Growth of* Bordetella pertussis *in* 0·1 % *Casamino Acids with haemin and iron salts*

Cultures incubated at 35° in 20 ml. CW3 (0·1 % w/v Casamino Acids). $FeSO_4.7H_2O$ (12·5 µg./ml.) was autoclaved in the medium when Seitz-filtered or autoclaved haemin was added but not when Seitz-filtered $FeSO_4$ was added to the autoclaved medium. $FeSO_4$ was sterilized by Seitz-filtration in acid solution; haemin was sterilized by filtration through a Gradocol membrane in alkaline solution. Strain 467 inoculum *c.* 10^3 bacteria/ml.

			Dry wt. (mg./ml.). Incubation time (hr.)					
Compound	Concentration (µg./ml.)	Method of sterilization	63½	72	89½	112	120	240
$FeSO_4.7H_2O$	12·5	Autoclaving	—	—	—	—	—	<0·01
	13·3	Filtration	0·025	—	—	0·50	—	—
	1·33	Filtration	—	—	—	—	—	<0·01
Haemin	2·5	Autoclaving	—	—	0·045	—	—	—
	2·5	Filtration	—	0·035	—	—	0·475	—
	0·25	Filtration	—	—	—	—	—	<0·01

Effect of iron. Small inocula grew in the medium containing 0·1 % (w/v) Casamino Acids (CW3) when ferrous sulphate sterilized by filtration was added to the medium after autoclaving (CW4). The lag period with 12·5 µg. $FeSO_4.7H_2O$/ml. was approximately the same as that with 1/10,000 red blood cells (Fig. 3); with lower concentrations of $FeSO_4$, the lag period was longer, and concentrations below 1 µg./ml. usually did not give growth.

The medium already contained $FeSO_4$ autoclaved with the other ingredients, but increasing the concentration of autoclaved $FeSO_4$ did not allow growth in CW3, and the autoclaved $FeSO_4$ could be left out without increasing the concentration of filtered $FeSO_4$. When it was sterilized by filtration, ferrous ammonium sulphate, which contains less impurity than $FeSO_4$ (analytical reagent grade), stimulated growth as well as $FeSO_4$.

Action of supplements. It was thus possible to obtain growth in a medium with 0·1 % (w/v) Casamino Acids by the addition of red blood cells, filtered ferrous sulphate, haemin, or by increasing the Casamino Acids concentration to 1 % (w/v). It was unlikely that haemin was the true growth factor because it was the least effective of the substances and Casamino Acids in 1 % (w/v) solution contained less than 0·01 μg. haemin/ml. (Professor C. Rimington, private communication). The lowest concentration of $FeSO_4$ which allowed growth contained 0·2 μg. Fe/ml. Vitamin-free Casamino Acids in 1 % (w/v) solution contained only 0·08 μg. Fe/ml. Bacto-Casamino Acids (iron-free) for the growth of *Corynebacterium diphtheriae* contained 0·0002 μg. Fe/ml. 1 % (w/v) solution, but gave only slightly slower growth of *Bordetella pertussis* than the vitamin-free hydrolysate: a 0·7 % (w/v) solution of the iron-free hydrolysate gave a visible turbidity at the same time as a 0·5 % (w/v) solution of the vitamin-free hydrolysate. If the stimulation by these two hydrolysates had been due to iron salts, the effect of the iron-free hydrolysate would have been relatively much less.

Ferrous sulphate and haemin are both reducing agents, and it was thought that the redox potential of the medium might be important. An attempt was made to measure it with phenolindo-2:6-dichlorophenol which has an E_0' at pH 7·0 of +0·217 V. Cysteine which did not promote growth with 0·1 % (w/v) Casamino Acids reduced the dye instantaneously. Ferrous sulphate which did promote growth reduced the dye slowly. Vitamin-free Casamino Acids reduced it slightly more quickly and Casamino Acids (iron-free) had very little effect on it. The result with cysteine suggests that the redox potential is not the factor governing growth.

Peroxides have been suggested as inhibitors of the growth of some organisms from small inocula (Gilder & Granick, 1948; Herbert, 1949), and ferrous ammonium sulphate and haemin permitted growth of *Haemophilus parainfluenzae* in such circumstances (Gilder & Granick, 1948). Ferrous sulphate, haemin and blood all destroy hydrogen peroxide, but permanganate titration showed that cysteine which did not support growth destroyed hydrogen peroxide rapidly. The destruction of hydrogen peroxide by Casamino Acids was not tested.

The active substance in Casamino Acids (vitamin-free) was partially removed by monochloroacetic acid + ether and the longer it remained with the monochloroacetate before the ether extraction the less activity was left. Passing through a column of Amberlite IRA 400 treated with monochloroacetate (see Schramm, Moore & Bigwood, 1954) and extracting with ether removed it completely but it is possible that this was merely the result of prolonged treatment with monochloroacetate.

Effect of various factors on growth rate

None of the factors mentioned in the preceding section had very much effect on the mean generation time (M.G.T.) which was usually 2·3–2·8 hr. Addition of lysed red blood cells to media with 1 % or 0·1 % (w/v) Casamino Acids usually decreased the M.G.T. slightly but only by about 0·5 hr. (Figs. 1, 3). This effect might be greater with an inoculum from a blood-free medium such as charcoal Cohen agar (see Mazloum & Rowley, 1955). When nicotinamide replaced yeast extract in medium CW 4 turbidity appeared later and with Casamino Acids left out as well growth was still slower (Table 3), but it is not known which phase of growth was affected. In my hands Proom's (1955) medium which contains 8 amino acids, starch, nicotinamide and salts gave very little multiplication and even with yeast extract added the M.G.T. was slower than that with medium CW 1. The medium of Jebb & Tomlinson (1955) containing charcoal-treated Casamino Acids, nicotinamide, purines, biotin and haemin gave very irregular slow growth. These two are the only simple media in which growth rates have been measured.

Table 3. *Effect of nicotinamide on growth of* Bordetella pertussis

Cultures grown at 35° in CW 2 with Casamino Acids, iron and yeast extract as below. Haemin was sterilized by autoclaving in the medium, $FeSO_4$ was sterilized by Seitz-filtration and was not added before autoclaving. The flasks with haemin contained iron only as impurity. Nicotinamide ($5\mu g./ml.$) was autoclaved in the medium. Strain 467, inoculum *c.* 10^3 bacteria/ml.

Additions			Dry wt. (mg./ml.). Incubation time (hr.)				
Casamino Acids	Growth factor	Iron source	63	139½	166	183	231½
0·1 %	Yeast extract	$FeSO_4.7H_2O$ (12·5 μg./ml.)	0·04	0·50	—	—	—
0·1 %	Nicotinamide	$FeSO_4.7H_2O$ (12·5 μg./ml.)	—	0·35	—	0·55	—
0·1 %	Nicotinamide	Haemin (2·5 μg./ml.)	—	0·20	—	0·475	—
0	Nicotinamide	$FeSO_4.7H_2O$ (12·5 μg./ml.)	—	—	0·03	—	0·375
0	Nicotinamide	Haemin (2·5 μg./ml.)	—	—	—	0·325	0·475

Secondary growth rate. Although the initial growth rate in CW media was always about the same, the time taken to reach a given turbidity was varied by the presence of a secondary slower logarithmic growth rate. This occurred in all experiments tested with 0·1 % (w/v) Casamino Acids + $FeSO_4$ medium (CW 4) and in almost all with 0·1 % (w/v) Casamino Acids + lysed red blood cells. In the experiment shown in Fig. 3, the M.G.T. fell slightly earlier in the medium + $FeSO_4$ than in that with lysed red blood cells so that after 63 hr. the dry weight with red blood cells was 4 times that with $FeSO_4$. With media containing 1 % (w/v) Casamino Acids (CW 1 or CW 2), the secondary growth rate was present in about 1/3 of the experiments; in the rest, either the primary growth rate was maintained to the end or data were insufficient to give the exact course of the curve. With this medium, there was some correlation between size of inoculum and presence of secondary growth rate: in ten experiments with a single logarithmic growth rate the inoculum was between 5×10^2 and 4×10^3; in six experiments with 2 logarithmic growth rates, four had an inoculum between 1×10^2 and 5×10^2 and two an inoculum of 1×10^3 bacteria/ml.

The secondary M.G.T. in medium CW 1 was generally between 3·1 and 3·8 hr.; in medium CW 3 with red blood cells or $FeSO_4$ it was between 4 and 7 hr. The change occurred at a viable count of between 10^6 and 10^8 and could not be explained by change in pH value, lack of glutamate, excess of ammonia (Table 4 and Fig. 1) or lack of oxygen. The aeration rate was not limiting until 0·1 mg. dry weight bacteria/ml. was present (see below).

The addition of 1/100 lysed red blood cells to medium CW1 kept growth at the primary rate throughout (Fig. 1). A secondary growth rate was present with 1/10,000 red blood cells and 0·1 % (w/v) Casamino Acids (Fig. 3), and the appearance of turbidity was slower in this medium than with 1/1000 red blood cells with 1 % (w/v) Casamino Acids (Table 6), suggesting that the primary growth rate went on for longer with 1/1000 red blood cells. The optimal concentration of red blood cells is not known.

Table 4. *Medium composition at start of secondary M.G.T.*

Data as in Fig. 1 for filtered cysteine (CW1).

Time of incubation (hr.)	Dry wt. of bacteria (mg./ml.)	pH	Glutamate (µl./ml.)		Ammonia (µl./ml.)	
			Total	Used	Total	Formed
0	—	6·90	106	—	129	—
53¼	0·02	6·89	—	—	124	−5
63½	0·085	7·20	95	11	134	5

pH. The only difference in growth noted between pH 6·9 and 7·7 was that the secondary growth rate at pH 7·5–7·7 was slightly slower than at the lower pH, for example 4·2 instead of 3·7 hr. In one experiment after 65 hr. incubation the dry weight of bacteria at pH 7·7 was only 0·01 mg./ml. while that at the lower pH values was 0·10 mg./ml. Unfortunately the media used in these experiments did not contain extra glutamate and the total crop of bacteria at each pH was only 0·5–0·6 mg./ml. which may have been too small to show an effect of pH on the total yield of organisms.

Effect of aeration. Experiments on aeration were carried out in large-scale cultures with large crops of bacteria. The medium used contained 1 % (w/v) casein hydrolysate and 0·1 % (w/v) glutamate (LS 1). Different volumes of culture medium in penicillin flasks were shaken on a shaker with shelves moving at three different rates (Kantorowicz, 1951). In the largest volume of culture (500 ml.) shaken at the slowest rate growth was the same as in the other flasks until a dry weight of 0·15 mg./ml. was reached and then the growth rate became progressively slower (Fig. 4) as each increase in dry weight made more oxygen necessary.

Growth rate and turbidity. The growth rate as measured by viable count and by turbidity was approximately the same until the maximum turbidity was reached (Fig. 5). The growth rate as measured by turbidity was never as rapid as the primary growth rate; by the time a visible turbidity was present, either the primary growth rate had started to fall (curve A), or the secondary growth rate was operating (curve B). *Bordetella pertussis* may be different from other organisms, but these findings suggest that the growth rate of bacteria in the visible range should not be taken as indicative of that at an earlier stage.

The total count was not measured and the turbidity of the American N.I.H. '*Haemophilus pertussis*' vaccine standard of 10,000 × 10^6 organisms/ml. was approximately equivalent to 0·2 mg. bacteria/ml. of a 72 hr. large-scale culture. Assuming this to be correct the viable count was only 20 % of the total, a much lower value than has been found by other workers (see Monod, 1949). Although these values may not be strictly accurate it is probable that the American standard is not thick enough for the number of bacteria it is said to represent.

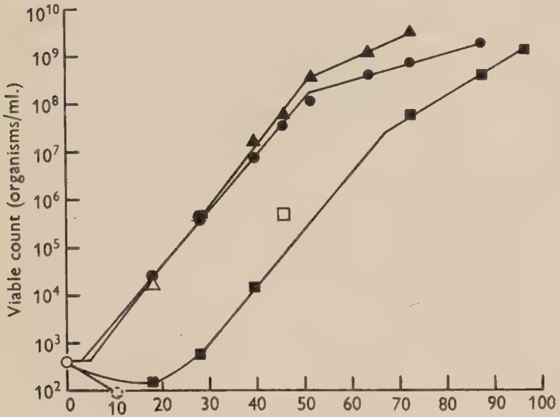

Fig. 3

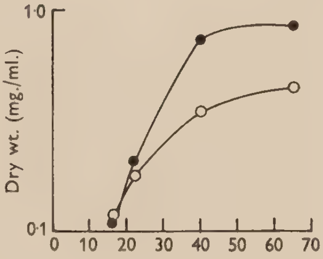

Fig. 4

Fig. 3. Stimulation of growth in 0·1 % (w/v) Casamino Acids by R.B.C., haemin and $FeSO_4$. Cultures incubated at 35° C. in 20 ml. medium CW3 with 0·1 % (w/v) Casamino Acids and additions as stated. Strain 467. CW3 without addition, ○—○; with 1/10,000 lysed R.B.C., ▲—▲; with haemin (2 μg./ml.), autoclaved in the medium, ■—■; with $FeSO_4.7H_2O$ (12·5 μg./ml.) sterilized by Seitz-filtration, ●—●. The number of colonies in the samples marked by the open square and the open triangle was too great to count accurately, and that in the dotted circle was too small.

Fig. 4. Effect of aeration rate on rate of growth. Cultures incubated at 35° in stated volumes of medium in penicillin flasks and shaken slowly. Medium LS1 containing 1 % (w/v) Benger's Casein hydrolysate. Strain 467. Inoculum *c.* 10^7 bacteria/ml. 500 ml. medium/flask, ○—○; 250 ml. medium/flask, ●—●.

Factors affecting the yield of bacteria

Glutamic acid. It was shown earlier (Rowatt, 1955) that the main amino acid used in growth of *Bordetella pertussis* was glutamic acid. The addition of more glutamic acid to a medium containing 1 % (w/v) Casamino Acids (CW2) increased the crop of bacteria, the maximum being 1 mg. bacteria/ml. with a total concentration of 0·2 % (w/v) glutamic acid (0·07 % in 1 % (w/v) Casamino Acids and 0·13 % (w/v) glutamic acid added). In this medium, the utilization of other amino acids was suppressed, only glutamate and alanine being used to a sufficient extent to show on paper chromatograms. At concentrations of glutamic acid above 0·2 % (w/v) growth stopped before all the glutamate had been used, showing that another factor was limiting.

In experiments with 500 ml. medium/flask and an inoculum of 10^7 organisms/ml., crops of 2·0–2·5 mg./ml. could be obtained. The crop of bacteria was proportional to the concentration of glutamate up to 0·3 % (w/v) giving approximately 1 mg. bacteria/2 mg. glutamate (Fig. 6). The mean generation time as measured by turbidity was approximately 5 hr. with concentrations of glutamate up to 0·5 % (w/v), but with 1 % (w/v) glutamate it was reduced to 8 hr. or more. The medium used for these experiments (LS 2) contained, instead of Casamino Acids, a pancreatic digest of casein (Benger Laboratories Ltd., Holmes Chapel) in which the concentration of free amino acids in a 1 % (w/v) solution was approximately the same as that in 0·1 % (w/v) Casamino Acids. The difference in yield of organisms was not caused by the alteration in amino acid source because similar crops were obtained from Casamino Acids when large inocula were used, although the rate of growth was sometimes slower.

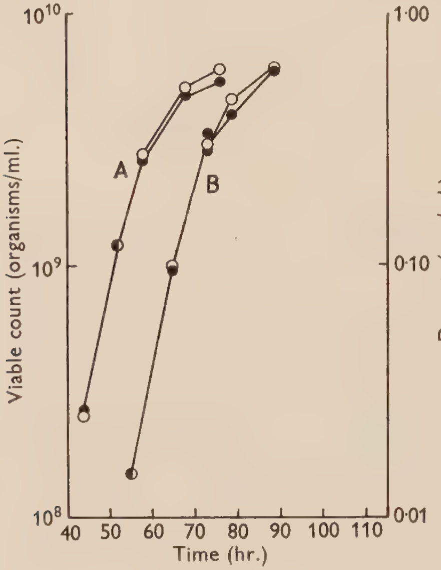

Fig. 5

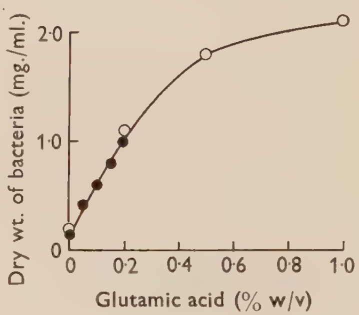

Fig. 6

Fig. 5. Comparison of growth rate as measured by viable count and by turbidity. Both cultures incubated at 35° in 20 ml. medium CW 1. A: strain 5339; inoculum 4×10^3 bacteria/ml.; primary M.G.T. ended at 44 hr. B: strain 951; inoculum $1·5 \times 10^2$ bacteria/ml.; primary M.G.T. ended at 41 hr., secondary M.G.T. ended at 65 hr. Viable count, ●—●; dry wt., ○—○.

Fig. 6. Effect of glutamic acid on growth in large-scale experiments. Cultures incubated at 35° C. in 500 ml. medium LS 2 containing 1 % (w/v) Benger's casein hydrolysate and stated concentration of glutamic acid. Final crop plotted for each concentration after 40–72 hr. incubation. Inocula *c.* 10^7 bacteria/ml. Strain M 2400, ●—●; Strain 467, ○—○.

During the growth of *Bordetella pertussis* glutamate was utilized with the formation of ammonia and a consequent increase in pH; in medium CW 1 approximately 1 ml. of 0·01 N-alkali was formed during the growth of 1 mg. bacteria. In the large-scale medium (LS 2) growth was never obtained at a higher pH than 8·2–8·3 even with 1 % (w/v) glutamate, and this pH is probably the limiting value. After growth had stopped the pH of the medium rose further, sometimes to pH 9·0, either by oxidation of glutamate or its breakdown products or by deamination of other amino acids.

Reducing the concentration of Casamino Acids in small-scale media decreased the yield of bacteria slightly (from 1·0 mg./ml. to 0·7–0·8 mg./ml.) even though extra glutamate was added. In media containing 0·1 % (w/v) Casamino Acids + haemin or + $FeSO_4$ (CW 4) the bacteria formed a scum on the medium which crept up the sides of the vessel. This did not happen in the medium with 0·1 % (w/v) Casamino Acids + lysed red blood cells or with 1 % (w/v) Casamino Acids, and it is probable that the medium + $FeSO_4$, although it supports growth, does not produce normal bacteria.

Cysteine or cystine is necessary for growth (Hornibrook, 1939). In large-scale experiments leaving out the casein hydrolysate (LS 3) reduced the yield of bacteria to approximately 0·8 mg./ml. or 1/3 of that with casein hydrolysate (LS 2). Preliminary experiments suggested that the crop could be doubled by using filtered instead of autoclaved cysteine (0·03 mg./ml.) (LS 4), but growth was not increased further with more cysteine. This quantity of growth was obtained with 0·06 mg. autoclaved cysteine/ml. (LS 3) and higher concentrations of autoclaved cysteine were inhibitory. It is possible that in the presence of casein hydrolysate the cysteine is more stable and less of the inhibitory breakdown products is formed.

Apart from glutamic acid and cysteine only alanine was used in any quantity during the early stages of growth. This could be seen from chromatograms of cultures in large-scale experiments in 0·5 % (w/v) glutamate without any casein hydrolysate (LS 3). Alanine was not an essential amino acid because the alanine spot on the chromatogram frequently became darker during growth indicating a synthesis of alanine. Further, its addition in the DL-form to medium LS 3 did not increase growth.

Not all of the nitrogen of the glutamate used appeared as ammonia. The missing nitrogen was not sufficient to account for the bacterial nitrogen and some must have come from other amino acids, but since the bacterial nitrogen and the non-glutamate nitrogen were of the same order and some amino acids remained after growth, a substantial part of the bacterial nitrogen must have come from glutamate (Table 5). The bacterial nitrogen was found to be 10 % of the dry weight, rather than the 12 % quoted previously (Rowatt, 1955).

Table 5. *Source of bacterial nitrogen*

Culture incubated at 35° 40 hr. in 500 ml. LS 3 0·5 % (w/v) glutamic acid without casein hydrolysate. Bacterial N taken as 10 % of dry wt. Strain 467. Inoculum 2×10^7 bacteria/ml.

Dry wt. (mg./ml.)	Bacterial N (μmole/ml.)	Glutamate used (μmole/ml.)	Glutamate used minus NH_3 formed (μmole/ml.)	Initial amino N other than glutamate and NH_3 (μmole/ml.)
0·70	5	12·8	4·9	4·7

Growth factors. The replacement of yeast extract by nicotinamide in medium CW 4 with 0·1 % (w/v) Casamino Acids + $FeSO_4$, although it made growth become visible later, did not alter the crop of bacteria significantly

(Table 3). The yield with yeast extract and 1/10,000 lysed red blood cells was no better but when a large inoculum was used in the medium with 0·1 % (w/v) Casamino Acids without red blood cells on a large scale, the yield of bacteria was of the order of 1·5 mg. dry weight/ml.

Autoclaved cysteine and 0·1 % *(w/v) Casamino Acids in growth of other Bordetella*

Autoclaved cysteine slowed the growth of Pollock's (1947) 'Pertussis A' and this strain would not grow in 0·1 % (w/v) Casamino Acids unless blood was added (Table 6). Phase IV CN 2217 and NCTC 364, which grow on digest agar, grew slowly with autoclaved cysteine but not with 0·1 % (w/v) Casamino Acids. Autoclaved cysteine did not affect the growth of G146E but with 0·1 % (w/v) Casamino Acids growth was slower. G146E and G154E both grew poorly in CW1. *Bordetella bronchiseptica*, NCTC 8762, grew at the same rate in all the media, and gave a smaller crop with 0·1 % than with 1 % (w/v) Casamino Acids. *B. parapertussis* grew at the same rate in the media without blood and seemed to be unaffected by autoclaved cysteine or by 'peroxides' but a visible turbidity appeared sooner when blood was added. Unfortunately, the effect of filtered $FeSO_4$ was not tested with any of these organisms.

DISCUSSION

Experiments on the growth requirements of *Bordetella pertussis* have always been complicated by the interplay of inhibitors. Two of these have been identified, namely colloidal sulphur or sulphides (Proom, 1955) and unsaturated fatty acids (Pollock, 1947). With the first of these the viable count remains stationary showing that division of the bacteria is inhibited. The main source of colloidal sulphur is autoclaved cysteine or cystine and can be eliminated by sterilizing these compounds by Seitz filtration. A small quantity of inhibitor may be introduced by protein hydrolysates or yeast extract. These can be sterilized by filtration, or autoclaved at acid pH or in the presence of phosphate or citrate to chelate heavy metals which accelerate the formation of colloidal sulphur or sulphides (Dr D. Herbert, private communication). Unsaturated fatty acids can be excluded by capping containers instead of plugging with cotton wool and by using a protein hydrolysate such as Casamino Acids which is low in fatty acids as estimated by *Corynebacterium diphtheriae* (Rowatt, unpublished experiments).

The nature of the third inhibitor is not yet established, but its removal by lysed red blood cells, haemin and filtered ferrous sulphate suggests that it might be a peroxide. The quantity of inhibitor as estimated by the amount of substance necessary to initiate growth varied with the batch of medium. The variable factor could not be the inoculum because in general three strains of organism were used with each batch of medium and each strain behaved in the same way with any one batch. This leaves the autoclaving of the medium as the cause of the inhibition. Peroxides are formed when media are

Table 6. *Growth of other Bordetella strains in autoclaved cysteine* (*CWa*) *and* 0·1 % (*w*/*v*) *Casamino Acids* (*CW*3)

Cultures incubated at 35° in 20 ml. medium. CW1 contained 1 % (w/v) Casamino Acids and either Seitz-filtered cysteine or cysteine autoclaved in the medium (CWa). CW3 contained 0·1 % (w/v) Casamino Acids and Seitz-filtered cysteine. Inocula *c.* 10^3–10^4 bacteria/ml.

	CW1 Cysteine						CW3			
	Filtered		Autoclaved		Autoclaved + 1/1000 R.B.C.		Alone		With 1/10,000 R.B.C.	
Organism	Time (hr.)	Dry wt. (mg./ml.)	Time (hr.)	Dry wt. (mg./ml.)	Time (hr.)	Dry wt. (mg./ml.)	Time (hr.)	Dry wt. (mg./ml.)	Time (hr.)	Dry wt. (mg./ml.)
B. pertussis, phase I M2400	66½	0·04	288	<0·01	42	0·15	288	<0·01	66½	0·14
‘Pertussis A’	66½	0·075	218	0·04	42	0·15	288	<0·01	42	0·13
Phase IV CN2217	47	0·06	111	0·475	40	0·075	135	<0·01	47	+
NCTC 364	66½	0·13	114½	0·70	42	0·125	288	<0·01	66½	0·25
G146E	42	0·02	66½	0·045	42	0·10	114½	0·035	114½	0·030
B. parapertussis, NCTC 8250	43	0·375	43	0·10	22	0·10	43	0·10	22	0·045
B. bronchiseptica, NCTC 8762	22	0·475	22	0·70	22	0·65	22	0·25	—	—

autoclaved at neutral pH, and hydrogen peroxide has already been suggested as an inhibitor of *Bordetella pertussis* on solid media (Mazloum & Rowley, 1955). However, since haemin destroys hydrogen peroxide more rapidly than $FeSO_4$ does, but is less active in promoting growth, it is more probable that an organic peroxide is to blame. This question could be settled by the addition to the medium of catalase which destroys hydrogen peroxide but not other peroxides. Until the identity of this third inhibitor is established, it is not possible to know the easiest way of overcoming it. However, it should be borne in mind that organic peroxides formed from ethers or alcohols during heating might be responsible for inhibitory effects such as that mentioned on p. 286.

Bordetella pertussis grew rapidly only in the early stages of growth or in media containing high concentrations of lysed red blood cells. Since inocula were grown on a medium rich in blood (BG), the initial rapid growth rate may have been due to factors carried over in the inoculum. Small quantities of the substances concerned may have been present in Casamino Acids because the secondary growth rate was present always in media containing 0·1 % but only sometimes in media containing 1 % (w/v) Casamino Acids. The variation with 1 % (w/v) Casamino Acids shows that most of the factor is added with the inoculum which is the main variable. The slower secondary growth rate of *B. parapertussis* (Rowatt, 1955) is probably due to the same factor especially as growth of this organism became visible sooner when lysed red blood cells were added to the medium. *B. parapertussis* which was maintained on media without blood (DA) would be expected to show deficiency of a factor in blood more readily than *B. pertussis* which was maintained on media rich in blood (BG). Factors in yeast extract and Casamino Acids probably affected the growth rate of *B. pertussis* rather than the lag period, making the factors in the inoculum unable to maintain the rapid growth rate.

Glutamic acid and the buffering capacity of the medium are the major factors governing the yield of *Bordetella pertussis* in a medium. Cysteine and unidentified factors in Casamino Acids are also necessary, but one of the most important factors is one added to the culture medium with large inocula. This factor or group of factors doubles or trebles the yield of bacteria and must be at least partially identified before large crops of *B. pertussis* can be obtained in simpler media than those described here.

Proom (1955) found that *Bordetella parapertussis* and *B. bronchiseptica* are not inhibited by colloidal sulphur. I have shown that they are not inhibited by 'peroxide'. However, the phase IV strains except G146E were all inhibited by the 'peroxide' medium and to some extent by autoclaved cysteine. G146E may have been slightly inhibited by autoclaved cysteine; in the 'peroxide' medium it grew very little, even with blood. It is not certain that the slowing of growth in 0·1 % (w/v) Casamino Acids (CW3) was due to the same factor for each strain. The difference between these five derived strains is emphasized by these tests.

Bordetella pertussis requires blood to combat inhibitions by 'peroxide' and by autoclaved cysteine and to maintain the growth rate. The greater yield of bacteria when a large inoculum is used may be caused by some of the constituents of blood added with the inoculum. This organism has been grown in defined media but rapid and profuse growth cannot yet be obtained without blood, and for some time to come the most complete media will probably contain small concentrations of blood added either as such or in the inoculum.

This work was carried out while the author was seconded from the Central Public Health Laboratory, Colindale.

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REFERENCES

Cohen, S. M. & Wheeler, M. W. (1946). Pertussis vaccine prepared with phase-1 cultures grown in fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

Fisher, S. (1948). The behaviour of *Haemophilus pertussis* in casein hydrolysate broth. *Aust. J. exp. Biol. med. Sci.* **26**, 299.

Gilder, H. & Granick, S. (1948). Studies on the *Haemophilus* group of organisms. Quantitative aspects of growth on various porphin compounds. *J. gen. Physiol.* **31**, 103.

Greaves, R. I. N. (1944). Centrifugal vacuum freezing. Its application to the drying of biological materials from the frozen state. *Nature, Lond.* **153**, 485.

Herbert, D. (1949). Studies on the nutrition of *Pasteurella pestis*, and factors affecting the growth of isolated cells on an agar surface. *Brit. J. exp. Path.* **30**, 509.

Hornibrook, J. W. (1939). Cultivation of phase I *H. pertussis* in a semi-synthetic liquid medium. *Publ. Hlth Rep., Wash.* **54**, 1847.

Hornibrook, J. W. (1940). Nicotinic acid as a growth factor for *H. pertussis. Proc. Soc. exp. Biol., N.Y.* **45**, 598.

Jebb, W. H. H. & Tomlinson, A. H. (1955). The nutritional requirements of *Haemophilus pertussis. J. gen. Microbiol.* **13**, 1.

Kantorowicz, O. (1951). Shaking apparatus for the aeration of bacterial cultures. *J. gen. Microbiol.* **5**, 951.

Leslie, P. H. & Gardner, A. D. (1931). The phases of *Haemophilus pertussis. J. Hyg., Camb.* **31**, 423.

Mazloum, H. A. & Rowley, D. (1955). The growth requirements of *Haemophilus pertussis* on solid media. *J. Path. Bact.* **70**, 439.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Monod, J. (1949). The growth of bacterial cultures. *Annu. Rev. Microbiol.* **3**, 371.

Pollock, M. R. (1947). The growth of *H. pertussis* on media without blood. *Brit. J. exp. Path.* **28**, 295.

Proom, H. (1955). The minimal nutritional requirements of organisms of the genus *Bordetella* López. *J. gen. Microbiol.* **12**, 63.

Rimington, C. (1942). Haemoglobinometry. *Brit. med. J.* i, 177.

Rowatt, E. (1955). Amino acid metabolism in the genus *Bordetella. J. gen. Microbiol.* **13**, 552.

Schramm, E., Moore, S. & Bigwood, E. J. (1954). Chromatographic determination of cystine as cysteic acid. *Biochem. J.* **57**, 33.

SMITH, J. C. (1939). Higher aliphatic compounds. VIII. Purification of oleic and elaidic acids. Binary systems from oleic, elaidic, palmitic and stearic acids. The technique of low temperature crystallization. *J. chem. Soc.* p. 974.

THORP, R. H. (1941). A method for the microestimation of iron in biological materials. *Biochem. J.* **35**, 672.

WOIWOD, A. J. (1954). The inhibition of bacterial growth by colloidal heavy metal sulphides and by colloidal sulphur. *J. gen. Microbiol.* **10**, 509.

W.H.O. Report (1953). Diphtheria and pertussis vaccination. *World Hlth Org. techn. Rep. Ser.* no. 61.

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ROWATT, E. (1957). *J. gen. Microbiol.* **17**, 297–326

The Growth of *Bordetella pertussis*: a Review

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INTRODUCTION

The existence of the organism causing whooping cough, *Bordetella pertussis*, was known for some years before it was grown on artificial media; after many attempts Bordet & Gengou (1906) obtained growth on a solid medium containing glycerinated potato extract and 50 % (v/v) blood. Simpler media were proposed but it was soon realized that an organism growing on these media differed antigenically from that originally isolated and until Hornibrook (1939) described a liquid medium containing amino acids, cysteine and starch, the Bordet-Gengou (BG) medium was the only one suitable for the isolation and continued cultivation of *B. pertussis*. With Hornibrook's and allied media the growth requirements of *B. pertussis* and its metabolism have been investigated by several workers and various inhibitors of its growth identified. Several solid media have been derived from the liquid medium with varying degrees of success, and progress has been made in primary isolation of the organism by the use of the bactericidal substance 4:4′-diamidinodiphenylamine dihydrochloride (M & B 938). Not all the chemical factors for rapid growth have been isolated and identified, and little is known of the organism's metabolism. Now that it is possible to grow the organism easily this should soon be remedied. This review deals with solid and then with liquid media for isolation and continued propagation, with the metabolism of the organism, followed by its relation to degraded strains, to other *Bordetella* and to other genera.

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SOLID MEDIA

Original isolation of the organism

Bordet & Gengou (1906) isolated the causative organism from the sputum of early cases of whooping cough on a glycerinated potato extract containing 50 % (v/v) rabbit or human blood, solidified with agar. Even on this medium, Bordet & Gengou noted that the organism grew feebly on first isolation. Later it grew more easily and by subculturing many times Klimenko (1908–09) obtained growth on ordinary nutrient agar. Such an organism Bordet & Sleeswyk (1910) showed to be antigenically different from freshly isolated strains. Later workers, not realizing this difference, modified the original medium by using heated blood (Krumwiede, Mishulow & Oldenbusch, 1923) or lower concentrations of blood (Debré, Marie & Pretet, 1928). Some workers, in particular Povitsky (1923) and Kristensen (1927), realized that such media were not suitable for isolation of the organism, but the position was not clear until Leslie & Gardner (1931) showed that freshly isolated strains were antigenically distinct from strains growing on simpler media. They found four agglutinogens and postulated the existence of four phases containing different combinations of them, phase I being the strain isolated from the patient and phase IV the most degenerate strain. Lawson (1939) could not find strains corresponding to phase II but confirmed the existence of phases I, III and IV. However, he also found a strain with characteristics of phases I and IV. Standfast (1951) showed that phase I and phase III or phase IV characteristics may be present in the same strain, that is, one strain may grow on 5 % (v/v) blood agar and be highly virulent to mice, while another is not virulent but will only grow on a Bordet-Gengou medium containing upwards of 20 % (v/v) blood. Differences of various derived strains will be mentioned later. This loss of virulence and alteration in growth requirements during subculture must be taken into account when media for the growth of *Bordetella pertussis* are tested, and any medium which will not grow *B. pertussis* on primary isolation should be discarded.

Modifications of BG medium

The various modifications of Bordet-Gengou medium have been mainly concerned with the time at which the glycerol is added, whether peptone is added, the pH value of the medium (before blood is added) and the addition of lactic acid. Bordet & Gengou (1906) extracted the potato with a glycerol solution. Other workers (see Mackie & McCartney, 1953) added glycerol after the extraction. According to Dawson, Farnworth, McLeod & Nicholson (1951) glycerol was inhibitory at concentrations above 1% (w/v) and could be omitted altogether without decreasing growth significantly. In earlier work glycerol was thought to be of value in slowing evaporation of the BG medium, but to control the humidity of the incubator is a more satisfactory way to prevent drying. Peptone was originally omitted from the medium to depress growth of other organisms present in sputum (Bordet & Gengou, 1906). Mackie & McCartney (1953) included peptone in their recipe and it was added to the W.H.O. medium for vaccine production (W.H.O. Report, 1953). However, Dawson *et al.* (1951) found all peptones to be inhibitory, although Witte peptone and Difco proteose peptone were less so than others. This inhibition is probably due to colloidal sulphur or sulphide (Woiwod, 1954; Proom, 1955). In view of the role of glutamate in the growth of *Bordetella pertussis* (see below) it would seem reasonable to add some source of this compound; in fact, Dawson *et al.* (1951) found that both glutamate and aspartate improved growth, and suggested that amino acids may be responsible for the effect of potato extract, of which the filtrate is the active fraction. Bailey (1933) substituted potato starch for some of the potato and claimed that growth was more rapid, but his evidence was scanty. Lacey (1954) also used starch instead of potato extract but he did not compare the two.

Effect of pH value and organic acids

Povitsky (1923) was the first to notice the effect of the pH value of solid media; she found that *Bordetella pertussis* grew better on media with a slightly acid reaction and that *Haemophilus influenzae* did not grow at this reaction although it grew slightly on the same medium at an alkaline reaction. The use of the acid medium, therefore, made primary isolation of *B. pertussis* easier. The pH value of the medium was tested before the addition of blood and must have been raised by the addition of blood, except in media initially at pH 7·2–7·4 which would be unchanged. Unadjusted BG base at pH 5·8–6·0 was first used; on it the isolation rate was higher than on the same medium adjusted to pH 7·2–7·4 before addition of blood. A still higher isolation rate was obtained by taking the pH value of the medium to 5·0 with 1/2500 acetic acid or to pH 4·5 with 1/2000 potassium oxalate. The acids were added after autoclaving since they would otherwise destroy the gelling power of the agar. Even so, material from only five out of seven patients in the first week of the disease yielded *B. pertussis*, and the small number of isolations detracts from the value of an otherwise excellent piece of work. It seems probable that the improvement in growth was due to the pH value but a specific effect of the anion was not excluded.

Madsen (1925) added about 1/2000 lactic acid to his medium. He adjusted the base to pH 7·0–7·5 and added blood and lactic acid after autoclaving. Leslie & Gardner (1931) also added lactic acid but neither of the last two papers records any comparison between media with and without lactic acid. Lacey (1954) used lactate to improve growth in a defined medium + blood which will be considered below; he also adjusted the pH value of the basal medium to 7·2 before adding blood, and most of the later recipes do this. In the experiments of Ungar *et al.* (1950) with liquid media, growth of *Bordetella pertussis* was better at higher pH values.

Media different from BG medium

Apart from the media containing charcoal, three media basically different from Bordet-Gengou medium have been described. Silverthorne & Cameron (1942) added 33 % (v/v) citrated sheep blood to a basal medium consisting of an aqueous extract of beef liver with salt and agar but without potato. They stated that growth was much more profuse and more rapid than on standard BG medium, but they did not give details. Since they subcultured at 48–72 hr. intervals it seems unlikely that growth was very much more rapid, because on good BG medium single organisms give visible colonies in 72 hr., and one normally uses a heavier inoculum than this for subculture; Dawson *et al.* (1951) found the medium no better than ordinary Bordet-Gengou medium.

Cruickshank & Freeman (1937) used 20 % (v/v) laked horse blood with 13 % (v/v) horse serum, proteose peptone and heart extract for growth of the organism in quantity; I found that, although growth on this media from a large inoculum might be more profuse than on BG medium, the number of colonies growing from a small inoculum of an exacting strain was generally only one-third and sometimes only one-tenth or a hundredth that on BG medium (Rowatt, unpublished). For large scale work it was not consistently better than BG medium (A.F.B. Standfast, personal communication).

The third medium was that of Barksdale & Simpson (1934; also see Barksdale, Mullenix & Fuster, 1941) which contained nutrient agar and potato extract and was reputed to give growth in 24 hr. and a ‘specific clearing’ in the medium in a shorter time. Dr B. W. Lacey (personal communication) was unable to grow *Bordetella pertussis* on this medium and my experience confirms his.

Media for isolation of Bordetella pertussis

The W.H.O. report on diphtheria and pertussis vaccination recommended for routine diagnostic purposes a modification of Bordet-Gengou medium, which they attributed to Eldering & Kendrick (W.H.O. Report, 1953). This medium contained 17 % sheep blood and peptone was not added. Penicillin is often added to the medium since *Bordetella pertussis* is relatively insensitive to this antibiotic, but the medium is still not completely satisfactory. Lacey (1954) devised a highly selective medium which contained more than 30 % (v/v) defibrinated horse blood, starch, glycerol and salts and, besides penicillin, M & B 938 and sodium fluoride. M & B 938 inhibited most other organisms of the nasopharynx very much more than it inhibited *B. pertussis*; the addition of magnesium ion and some organic acids decreased the inhibition of *B. pertussis* more than that of the other organisms. Some organisms, notably streptococci which are less susceptible to M & B 938, were inhibited by fluoride but this substance inhibited the growth of *B. parapertussis* so that an ordinary BG plate had to be used to detect it. Aconitate or lactate was added to improve the growth of *B. pertussis*; it is interesting that here we have a return to the work of Povitsky (1923).

Lacey's medium, although excellent for isolation is even more difficult to prepare than BG medium; it contains eleven extra compounds, and four solutions, including blood, must be added aseptically to the autoclaved base. Although its qualities are thoroughly appreciated it is no longer used by some laboratories because of the difficulty of preparation. The medium at present used by the Central Public Health Laboratory, Colindale (Mr J. D. Atkinson, personal communication) contains peptone, sodium chloride, glycerol, soluble starch and agar. These ingredients are sterilized together and to them are added horse blood to a final concentration of 33 % (v/v), penicillin and M & B 938. This medium gives satisfactory growth of *Bordetella pertussis* and *B. parapertussis*, and growth of most other organisms is suppressed by the penicillin and M & B 938. The organic acids and magnesium salts in Lacey's medium make *B. pertussis* much less sensitive to M & B 938 and, in their absence, the concentration of this compound must be carefully controlled. When this precaution is taken and sufficient blood is added, the exact composition of the medium is not very important. With Atkinson's medium the isolation rate is slightly lower than with Lacey's medium, but the greater ease of preparation and the fact that *B. parapertussis* will grow on it make it more suitable for routine work (Mr J. D. Atkinson, personal communication).

The elimination of blood

Blood and other solutions which must be added to the medium after autoclaving are a constant source of trouble, and several attempts have been made to eliminate blood from the medium. Pollock (1947) used a strain ('Pertussis A') which would grow on 10 % (v/v) blood agar but not on meat digest agar, retaining this growth characteristic through many subcultures. He found that for this strain blood in the solid medium could be replaced by serum, serum albumin or charcoal. Starch had some effect but was not so effective as albumin or charcoal. The unsaturated fatty acids, oleic, linoleic and linolenic, all inhibited the growth of 'Pertussis A' in solid and in liquid media, but the inhibition was overcome by albumin; Pollock suggested that overcoming this inhibition was the function of blood in the growth of the degraded strain. Serum albumin improves the growth of *Mycobacterium tuberculosis* in the presence of Tween 80 and Davis & Dubos (1946) showed conclusively that albumin binds inhibitory unsaturated fatty acids firmly and allows growth of the bacillus. 'Pertussis A' would not grow in nutrient broth, but when charcoal or albumin was added the organisms grew quite well. Pretreatment of the medium with charcoal was not enough; the charcoal had to be present during growth. When the charcoal from a medium in which 'Pertussis A' had grown was extracted with methanol, the extract inhibited growth of 'Pertussis A' on agar plates containing albumin, and the inhibitory power was approximately one-hundredth that of oleic acid. The inference is that during growth 'Pertussis A' forms a substance similar to oleic acid, and thus inhibits its own growth (Pollock, 1947). I have found oleic acid to be inhibitory to growth of *B. pertussis*, but it is certainly not the only inhibitor (see below). However, albumin and possibly charcoal also neutralize some of the other inhibitors.

A liquid medium solidified with agar would be a convenient substitute for BG medium. However, Verwey, Thiele, Sage & Schuchardt (1949) found that *Bordetella pertussis* would not grow in their medium solidified with agar unless blood were added; this is also true of Cohen & Wheeler's (CW) medium (Mazloum & Rowley, 1955). On the other hand, *B. pertussis* grew on CW agar containing 2·5 % (v/v) blood and either serum or red cells could be used at the same concentration, but growth was generally less profuse than on BG medium (Rowatt, unpublished). A preparation of serum albumin *c.* 95 % pure supported growth but electrophoretically pure serum albumin gave growth only from large inocula. Although CW agar + serum usually gave growth from small inocula, the CW agar culture was not always satisfactory as an inoculum for liquid CW. An inoculum from CW agar + blood, for example, gave visible growth after 3 days of incubation in liquid CW medium, whereas an inoculum from CW agar + serum took 4 days (Rowatt, unpublished).

Use of charcoal

Powell, Culbertson & Ensminger (1951) described a medium for growth of vaccine strains consisting of CW medium, agar and charcoal. Growth from large inocula was quite good and the vaccine produced was satisfactory (Ensminger, Culbertson & Powell, 1953). I found that CW agar + charcoal usually enabled *Bordetella pertussis* to grow from large inocula but not always from small ones. Norite S.G. which was used by Powell *et al.* (1951), Norite (Harrington Bros.) and activated charcoal (British Drug Houses Ltd.) all gave irregular results (Rowatt, unpublished). Mazloum & Rowley (1955), using Sutcliffe-Speakman charcoal No. 5, had to add an inoculum of 1×10^6 organisms/drop (technique of Miles & Misra, 1938) to obtain growth on this medium. Mishulow, Sharpe & Cohen (1953) used a medium of beef heart extract, peptone and yeast-extract agar to which was added 0·4 % (w/v) charcoal. They do not give details of yield of organisms but the vaccine they prepared compared well with their standard vaccine. Although the organism could be subcultivated on this medium several times without change of phase, the vaccine was made by growing an inoculum from Bordet-Gengou medium on small quantities of charcoal agar and then on larger quantities of the same medium. I was unable to grow *B. pertussis* on this medium from small inocula.

Addition of catalase

Mazloum & Rowley (1955), using a high grade charcoal (Sutcliffe-Speakman No. 5), found that single organisms of *Bordetella pertussis* did not grow on the medium of Powell *et al.* (1951). They found that *B. pertussis* sown on CW agar + charcoal (0·7 %, w/v) showed satellite growth near some but not all strains of *Staphylococcus aureus* (one-third of those tested) and certain other aerobic organisms. A cell-free preparation of *S. aureus* also stimulated growth, and although the staphylococcal factor would not diffuse through cellophan, growth occurred with the staphylococcal extract on one side of the cellophan and *B. pertussis* on the other. The extract could be replaced by crude catalase (5% pure) and 60% pure catalase at low concentrations, suggesting that growth was being inhibited by hydrogen peroxide. This compound has been implicated as an inhibitor of the growth of several organisms: for example, *Pasteurella pestis* needs haemin for aerobic but not anaerobic growth on a simple agar medium, suggesting that hydrogen peroxide inhibits growth aerobically (Herbert, 1949). It is probable that growth of *B. pertussis* in liquid media is inhibited by peroxides (Rowatt, 1957).

Mazloum & Rowley's (1955) medium for the growth of *Bordetella pertussis* consists of Cohen & Wheeler's medium with agar, charcoal and a catalase preparation. Catalase does not promote growth in the absence of charcoal (Rowatt, unpublished), although charcoal does to some extent in the absence of catalase (Mazloum & Rowley, 1955). As an ingredient of a medium catalase is easier to handle than blood, but it is heat labile and destroyed by hydrogen peroxide. A heat-stable compound such as haemin would simplify the making of the medium but this has not yet been tested.

To summarize, solid media for *Bordetella pertussis* have been developed with two different aims: (i) a medium for isolation such as the original BG, Povitsky's and Lacey's media; (ii) a medium for growing large quantities of organism, such as the media of Cruickshank & Freeman (1937), Mishulow *et al.* (1953) and Powell *et al.* (1951). Besides a high concentration of blood, isolation media generally contain: starch in some form although it has not been proved that this is necessary; a source of amino acids (potato extract, protein hydrolysate or the individual amino acids); and a device for making *B. pertussis* grow better than other organisms of the nasopharynx. This last was accomplished by Bordet & Gengou by omitting peptone, by Povitsky by lowering the pH value of the medium and by Lacey by adding penicillin + M & B 938 + organic acids. Lacey's method is the most effective. Mazloum & Rowley's medium with charcoal and catalase might be sufficient for primary isolation because it will grow *B. pertussis* from single organisms. It should be possible to obtain medium combining these recipes, able to give profuse growth with amino acids and possibly organic acids and suitable for isolation when bactericidal compounds are added.

LIQUID MEDIA

Bordetella pertussis was found not to grow in liquid media without blood (Lawson, 1939) or tissue extract (Toomey & McClelland, 1933–34). Such media could not be used for vaccine production for fear of stimulating the formation of antibodies to the other proteins and the bacteria are so much contaminated with protein that they are of little use for metabolic or other tests.

Hornibrook's medium

In 1939, Hornibrook described a medium for *Bordetella pertussis* which did not contain blood or tissue extract; it was made of hydrolysed casein, soluble starch, salts, cysteine and sometimes yeast extract. Liquid media have since been based on Hornibrook's recipe. In 1940 Hornibrook described another medium containing amino acids + starch + nicotinamide. Nicotinamide is the only firmly established essential growth factor for *B. pertussis*. Hornibrook's first medium was not entirely satisfactory and improvements were suggested. Many of the modifications were alterations of the growth factor source and were dictated by what was readily available locally. For example, Wilson (1945) and Farrell & Taylor (1945) both used liver extracts. Even so the latter authors obtained only $1{\cdot}5 \times 10^{10}$ organisms/ml. On the other hand, Verwey *et al.* (1949) used nicotinic acid instead of yeast extract and their crop was $2\text{–}5 \times 10^{10}$ organisms/ml. The number of bacteria in cultures as measured by comparison with the American N.I.H. '*Haemophilus pertussis*' vaccine standard of 1×10^{10} organisms/ml. was five times that found by viable count (Rowatt, 1957), and it is probable that the turbidity of the standard is too low. This should be borne in mind when yields of bacteria are compared, since the numbers quoted may have been measured by different methods.

Aeration

Hornibrook (1939) incubated cultures in bottles lying on their sides to expose a large surface of medium to the air; other workers have followed this advice, in some cases shaking the bottles to give a higher degree of aeration. Ungar *et al.* (1950) found that a large area of medium exposed to air increased the rate of growth of virulent strains and increased the yield of bacteria slightly, but bubbling air through the medium killed bacteria of a virulent strain, though it improved growth of an avirulent strain. Although they used an inoculum of $10^6\text{–}10^8$ organisms/ml. growth in their medium was very slow, continuing until the 10th to 15th day, and it is possible that during the aeration cysteine was oxidized to give colloidal sulphur or that some inhibitory peroxide was formed (see below). I found (Rowatt, 1957) that, until the organisms reached a concentration of 0·1 mg. dry wt./ml., the rate of shaking did not matter, but above this turbidity the degree of aeration limited the rate of growth; on the other hand rapid shaking was not inhibitory.

Cohen & Wheeler's modification

Cohen & Wheeler (1946) modified Hornibrook's (1939) medium and carried out potency tests on the vaccine obtained. This medium, recommended by the World Health Organization for preparation of Pertussis vaccines (W.H.O. Report, 1953), used Bacto-Casamino Acids (Difco) as source of nitrogen and this, being readily available and similar from batch to batch, gave a possible uniformity to the vaccines prepared. The sodium and calcium concentrations were decreased to make the cultures less granular. However, the decrease in sodium chloride to 0·25 % (w/v), together with 0·4 % (w/v) NaCl added in the Casamino Acids (technical) gave a final concentration of 0·65 % NaCl which is higher than that used by Hornibrook (1939).

Cohen & Wheeler increased the concentration of potassium phosphate to improve the buffering power and added ferrous and copper sulphates. The quantity of starch was also increased, but some would be removed when the medium was boiled and filtered. The yeast extract was a dialysate added with the cysteine before autoclaving. The inoculum was transferred from BG medium through increasing volumes of the liquid medium and finally to the vaccine batch giving a crop of *c.* $1–1\cdot5 \times 10^{10}$ organisms/ml.

This is one of the few growth media for *Bordetella pertussis* in which the salt concentrations were worked out experimentally. In most media the salt mixture is arbitrary and vaguely derived from physiological saline. It is unlikely that extra copper is necessary since some is present in Casamino Acids and omission of copper from this medium does not affect growth (Rowatt, unpublished). When making this medium, I have used magnesium sulphate instead of magnesium chloride because the latter is highly deliquescent when hydrated and cannot be dried to a compound of known degree of hydration.

Vaccine production

With an inoculum from liquid media we found that Cohen & Wheeler's medium could not be relied upon to give consistent yields of cells (A. F. B. Standfast and co-workers, unpublished). Hornibrook (1939) used an inoculum straight from BG medium but was able to grow the organisms through four subcultures in a liquid medium containing cysteine or glutathione sterilized by filtration. When the cysteine was autoclaved in a complex medium, except in certain ill-defined circumstances, it was necessary to use a large inoculum straight from BG medium to get a good crop (Rowatt, 1957). The large inoculum probably increased the yield of bacteria (Rowatt, 1957) and for routine vaccine production it is generally simpler to autoclave cysteine in the medium and inoculate with the growth from one BG slope/500 ml. medium, than to add cysteine after autoclaving and use a smaller inoculum. In this way the medium can be autoclaved entire and a contaminated slope will affect only one flask. The quantity of blood in the finished vaccine is negligible. Many workers recommend a high initial pH value (e.g. Ungar *et al.* 1950), but I have found (Rowatt, 1957) that the organism grows at the same rate between pH 7·0 and 7·5 and, since alkali is formed during growth, the starting medium should be as acid as possible, i.e. pH 7·0. As explained below, growth is increased by raising the concentration of glutamic acid in the medium. In this laboratory I have obtained crops of ten times the American N.I.H. *Haemophilus pertussis* vaccine standard of 10^6 organisms/ml. (2–2·5 mg. dry wt./ml.) in a medium with autoclaved cysteine containing 1 % Benger's tryptic casein hydrolysate + 0·5 % (w/v) glutamic acid (72 hr. incubation in 500 ml. medium) (Rowatt, 1957). Vaccines prepared in this medium gave as good protection in mice as routine vaccines (J. M. Horton, unpublished). The casein hydrolysate used, although less suitable for growth from small inocula, is more suitable than Casamino Acids for growth from large inocula, because the organisms are less inclined to autoagglutinate (Rowatt, unpublished). Growth is limited by lack of one or more factors and possibly by insufficient buffering power of the medium. The medium could probably be simplified by omitting copper sulphate. It is not perfect but will regularly give a large crop of bacteria in the antigenic phase desired.

The absolute growth requirements of Bordetella pertussis

The effect of starch

One of the most important factors in Hornibrook's original medium was starch. This had been used by Lederle Laboratories for some time for the growth of *Bordetella pertussis* (Hornibrook, 1939); Weichsel, Rubin, Cohen & Lapin (1940) reported its use in a meat infusion broth for the growth of a strain which grew on blood agar. Starch could be replaced by α- or β-dextrin (Hornibrook, 1939) or by amylose (Ungar *et al.* 1950). Hornibrook (1939) concluded that starch was broken down during growth of *B. pertussis*, because after growth the starch iodine colour was lighter than before, but Ungar *et al.* (1950) suggested that fatty acids produced by *B. pertussis* during growth caused the change in colour and that the starch was not used. In view of the apparent inability of *B. pertussis* to use simpler carbohydrates (see below) utilization of starch seems unlikely. Proom (1955) found that starch was necessary for growth of *B. pertussis* in a defined medium, although *B. parapertussis* and *B. bronchiseptica* grew in the same medium without starch. I found that in a modified Cohen & Wheeler's medium (CW 1) without starch, *B. pertussis* grew slowly and replicate flasks became turbid at different times, suggesting that so few organisms grew in each flask that a difference of one viable organism made a significant difference in the time taken to reach a visible turbidity (Rowatt, unpublished). This suggests that starch removes a factor which inhibits initiation and/or continuation of multiplication.

One function of starch in the medium, although probably not the main one, is to reduce precipitation of phosphates during autoclaving; in a medium autoclaved without starch or without Casamino Acids, a heavier precipitate forms. This is discussed below (p. 314).

Inhibition of growth by fatty acids

It has been suggested that starch annuls inhibition of growth by unsaturated fatty acids; the work of Pollock (1947) has already been mentioned. He obtained a degraded strain of *Bordetella pertussis*, 'Pertussis A', which grew on 5 % (w/v) blood agar but not on digest agar. The blood could be replaced by serum, serum albumin, charcoal or, less successfully, by starch. When incubated in broth 'Pertussis A' died in about 20 hr., but it grew when albumin was added and growth could be obtained when the albumin was added nearly 20 hr. after inoculation. The organism grew in broth from small inocula in the presence of charcoal or serum albumin; with starch growth was irregular unless a large inoculum was used. Unsaturated fatty acids inhibited growth in the presence of certain concentrations of albumin, but higher concentrations overcame the inhibition. This suggests that growth of this strain of *B. pertussis* in broth is prevented by unsaturated fatty acids.

Bordetella pertussis does not normally grow in Cohen & Wheeler's medium when a small inoculum is used. However, Fisher (1948) managed to obtain growth by treating the casein digest with fat solvents, 'Supercell' and 'Nuchar' and Jebb & Tomlinson (1955) obtained growth by heating the casein hydrolysate with charcoal at acid and neutral pH values. I found (Rowatt, unpublished) that the treatment used by Jebb & Tomlinson did not alter the 'oleic acid' content of Casamino Acids as estimated by Corynebacterium 'Q' (Pollock, Howard & Boughton, 1949). Corynebacterium 'Q' grew sparsely with either charcoal-treated or untreated Casamino Acids, and added oleic acid stimulated growth equally in each medium. Fisher's treatment probably removed fatty acids, but this need not have been the reason why growth occurred.

Pollock (1948) found that during autoclaving of media fatty material was extracted from the cotton wool plugs of the flasks or test tubes, and deposited on the glassware in quantity sufficient to inhibit growth of *Bordetella pertussis* in the volumes of media normally used. He overcame this difficulty by using for plugging glass wool extracted with methanol; other workers have used aluminium caps (Proom, 1955; Jebb & Tomlinson, 1955) or small glass beakers (Rowatt, 1955). Small inocula of *B. pertussis* in Cohen & Wheeler's medium + a filtered solution of cysteine grew readily in flasks capped with beakers (see below); with cotton-wool plugs growth was merely slowed down, not stopped altogether (Rowatt, unpublished). In this medium, oleic acid (25 μg./ml.) inhibited growth and the inhibition was not annulled by 1/1000 lysed unwashed red blood cells, an amount which allowed growth from small inocula in Cohen & Wheeler's medium + autoclaved cysteine, but may have been too little to combat this concentration of oleic acid (Rowatt, 1957). Pollock (1947) found that growth in broth sterilized in flasks with cotton-wool plugs (see Pollock, 1948) was inhibited by 4 μg. oleic acid/ml. when small concentrations of albumin (0·1–0·2 %, w/v) were present. In my medium sterilized in flasks capped with beakers, growth was not inhibited by 5 μg. oleic acid/ml. (Rowatt, 1957) but this medium contained very little of the fatty acid which supports the growth of Corynebacterium 'Q' (Rowatt, unpublished) and contained starch which may adsorb fatty acids. It is not surprising that oleic acid has less effect in this medium than in Pollock's (1947) medium.

As already mentioned (p. 301), Pollock (1947) obtained material inhibitory to 'Pertussis A' from charcoal which had been present during growth of the organism in broth; more of this material was obtained from broth in which 'Pertussis A' had grown than from uninoculated broth (Pollock, 1949), Corynebacterium 'Q', which requires oleic or other unsaturated fatty acid for growth, grew better in a mixed culture with 'Pertussis A' than in pure culture in the same medium; Corynebacterium 'Q' also allowed the growth of 'Pertussis A' in broth. This evidence suggested that 'Pertussis A' formed unsaturated fatty acids which inhibited its own growth but stimulated that of Corynebacterium 'Q' (Pollock, 1949). The formation of fatty acid during growth would cause a progressively increasing inhibition; I did not find this in any medium I tried and it seems probable that either the formation of unsaturated fatty acids did not occur or was suppressed in these media, or that the acids were picked up by the starch as soon as they were formed.

Inhibition of growth by sulphur compounds

In the Cohen & Wheeler type of medium starch permitted growth from large inocula but not from inocula of less than 1×10^6 organisms/ml. medium. Small inocula grew when the casein digest had been treated with charcoal (Fisher, 1948; Jebb & Tomlinson, 1955) or when albumin or blood was added to the medium (Fisher, 1948; Rowatt, 1957). Brucella strains would not grow in media containing Tryptose and the inhibition which could be removed by treating the Tryptose with Norite (Schuhardt, Rode, Oglesby & Lankford, 1950) was shown to be caused by elementary sulphur formed during the autoclaving of cysteine in the Tryptose (Schuhardt, Rode, Oglesby & Lankford, 1952). Woiwod (1954) showed that during the autoclaving of cysteine in complex media colloidal copper sulphide and colloidal sulphur were formed. Both sols inhibited the growth of *Staphylococcus aureus* and colloidal copper sulphide inhibited the growth of *Bacillus* and *Brucella* spp. Proom (1955) found that passage of H_2S through media containing mixtures of amino acids to remove heavy metals made the media inhibitory to growth of *Bordetella pertussis* from small inocula. Some casein hydrolysates did not allow growth of *B. pertussis* and this organism too was found to be inhibited by colloidal sulphur and to a lesser extent by colloidal copper sulphide. *Bordetella parapertussis* and *B. bronchiseptica* were not inhibited by colloidal copper sulphide and phase IV strains of *B. pertussis* were less susceptible than phase I (Proom, 1955). Two very rough strains of *B. pertussis* (G154E, G146E) were not inhibited by the quantity of autoclaved cysteine in Cohen & Wheeler's medium (Rowatt, 1957). Colloidal sulphur or sulphides may be responsible for the inhibitory effect of peptones in solid media (see Dawson *et al.* 1951).

In the Cohen & Wheeler type of medium colloidal sulphur formed from autoclaved cysteine was the chief inhibitor of growth of *Bordetella pertussis*. It acted by preventing multiplication of the inoculum; the organisms remained viable for *c.* 40 hr. and then began to die. With filtered cysteine they began to multiply after 5–18 hr. Cultures containing blood began to multiply slightly more quickly; for example, the lag period with blood might be 12 hr. and without, 16 hr. (Rowatt, unpublished), but the initial mean generation time was the same in the two cultures (Rowatt, 1957). Cultures containing filtered cysteine solution are therefore growing initially almost at maximum speed.

Inhibition caused by colloidal sulphur in the casein hydrolysate may have been overcome by the cystine or cysteine added (Woiwod, 1954; Proom, 1955); but growth was not slowed down in a medium without added cysteine except for the small quantity added with the yeast extract (Rowatt, 1957). It is possible, therefore, that the charcoal treatment of Casamino Acids used by Jebb & Tomlinson (1955) may have decreased the formation of inhibitor from autoclaved cysteine or lessened its effect. Growth was not inhibited when cysteine was autoclaved in a simple medium with glutamate as the only other amino acid (Jebb & Tomlinson, 1957).

Requirement for cysteine or cystine

Besides being the cause of inhibition of growth under certain conditions as described above cysteine is necessary for growth. Hornibrook (1939) showed that cysteine could be replaced by cystine or glutathione but not by methionine; Proom (1955) confirmed this by serial subcultivation. Cysteic acid, homocystine and sulphate are also inadequate (Jebb & Tomlinson, 1957). In experiments with large inocula, I found (Rowatt, 1957) that added cystine or cysteine increased the total crop of bacteria in media in which glutamate and yeast extract were the only source of nitrogen, i.e. without casein hydrolysate. With filtered cysteine solution, 30 mg. cysteine/l. final concentration gave the maximum yield of bacteria, but with cysteine autoclaved in the medium, twice this concentration was needed to give the same yield of bacteria and higher concentrations were inhibitory. Growth with filtered cysteine solution was restricted by lack of another factor.

Bactericidal effects in growth media

In the previous sections two inhibitors of the growth of *Bordetella pertussis* have been mentioned, namely unsaturated fatty acids and colloidal sulphur or sulphides. The effect of unsaturated fatty acids uncomplicated by other inhibitors has not been studied. Autoclaved cysteine, the source of colloidal sulphur and sulphides, has a bacteriostatic effect: the viable count did not fall until about 40 hr. after inoculation into the inhibitory medium. However, a third inhibitor (Rowatt, 1957) killed the bacteria rapidly: after 15 hr., in the presence of this inhibitor, the viable count had fallen to less than a tenth of the original. The inhibitor was found in media containing low concentrations of casein hydrolysate. The inhibition cannot be related to that caused by autoclaved cysteine; the two effects were different in type and substances which annulled one inhibition did not annul the other. The inhibition in low concentrations of Casamino Acids was overcome by red blood cells, but the quantity needed was less than that required to overcome the inhibition by autoclaved cysteine: in some experiments a final dilution of 1/100,000 red blood cells was sufficient. Casamino Acids apparently contained a factor similar to that in red blood cells and at concentrations greater than 0·3 % (w/v) this prevented some of the bacteria from being killed. Haemin had some activity and ferrous sulphate sterilized by filtration gave as short a lag period as red blood cells, but autoclaved ferrous sulphate had no effect. These compounds all prevented the lethal effect of some substance in the medium. When insufficient of these substances to give optimal growth was added the colony count fell, sometimes (especially with Casamino Acids or haemin) to a very low value, and the organisms were found to be growing at the normal logarithmic rate as soon as sufficient were present to be measured accurately. This inhibitor did not seem to affect the rate of growth once multiplication began.

The quantity of haemin, of red blood cells and of $FeSO_4$ necessary to start growth seemed to vary from one batch of medium to another. The variation with haemin was most marked: in some experiments 2·5 μg. haemin/ml. medium gave visible growth after 100 hr. and in others 0·25 μg. haemin/ml. gave visible growth in the usual time of 60 hr. Since the same Casamino Acid solution and extract of yeast were used for several batches of medium, the only variables were the inoculum and the autoclaving of the medium. It will be shown later that the size of inoculum may affect the rate of growth in later stages.

Hydrogen peroxide (Barry, Conalty, Denneny & Winder, 1956) and organic peroxides (Woiwod, private communication) may be formed during autoclaving of various media and hydrogen peroxide, and possibly some organic peroxides would be destroyed by blood, haemin or ferrous sulphate. *Bordetella pertussis* contains catalase (Portwood, 1946), but in the early stages of growth the catalase content of some organisms is low (Cole, quoted by Hinshelwood, 1946; Pinsent, quoted by Herbert, 1949). Thus, although hydrogen peroxide formed by the organisms could probably be destroyed, the catalase content might be too small to destroy a quantity of hydrogen peroxide such as would be formed during autoclaving of the medium. Catalase would not destroy an organic peroxide and inhibition by an organic peroxide seems more likely because cysteine which destroys hydrogen peroxide rapidly did not overcome the inhibition (Rowatt, 1957). However, Mazloum & Rowley (1955) used catalase to improve growth of *B. pertussis* on a solid medium. More work must be done to identify the inhibitor in liquid media.

The function of glutamic acid

Bordetella pertussis does not use any of the common sugars either aerobically or anaerobically (see, for example, Jebb & Tomlinson, 1951) and amino acids must be supplied for its growth. Glutamic acid is one of the amino acids used (Ungar *et al.* 1950) and when it has disappeared from the medium, growth stops (Jebb & Tomlinson, 1951). In the early stages of growth in a medium containing 1% (w/v) Casamino Acids and yeast extract, the quantity of ammonia formed was, mole for mole, considerably less than the quantity of glutamate used. Later, more ammonia was formed by the deamination of other amino acids. In these experiments (Jebb & Tomlinson, 1951) large inocula (1×10^7 organisms/ml.) were used and the yield of bacteria was at least three times that obtained by incubation of small inocula (1×10^2 organisms/ml.) in similar media (Jebb & Tomlinson, 1955). With large inocula (1×10^7 organisms/ml.) I also obtained better growth of bacteria (Rowatt, 1957) and a ratio of ammonia formed to glutamate used of 0·6–0·7 (Rowatt, unpublished). However, with small inocula (1×10^3 organisms/ml.) the ammonia formed was initially approximately equivalent to the amount of glutamate used. Increasing the glutamate concentration increased the crop slightly and suppressed the deamination of other amino acids, especially serine and aspartic acid.

When large inocula were incubated in a medium containing casein hydrolysate (Benger), the quantity of bacteria formed was proportional to glutamate concentration up to 0·3 % (w/v); the maximum yield (2·5 mg. dry wt./ml.) was obtained with 1 % (w/v) glutamate (Rowatt, 1957). During growth the bacterial-N of a culture was of the same order as the glutamate-N which did not form ammonia, both in the casein hydrolysate + glutamate medium and in a medium from which the casein hydrolysate was omitted, leaving yeast extract as the source of other amino acids. In the casein hydrolysate-free medium growth was limited by lack of a factor or factors present in the casein hydrolysate and stopped before all the glutamate had been deaminated. In this medium the bacterial-N was approximately equivalent to the non-glutamate amino-N, but according to paper chromatograms the concentration of amino acids, apart from glutamate and alanine, was not significantly altered during growth. The greater part of the bacterial-N must have come from glutamate (Rowatt, 1957). In the medium without casein hydrolysate, after growth had stopped, the remaining glutamate was deaminated with the same ratio of glutamate used to ammonia formed as during growth (Rowatt, unpublished). Paper chromatograms showed an increase in the alanine, valine and leucine spots during this period; unfortunately the amounts were not measured quantitatively nor these amino acids identified directly. Lack of certain amino acids or a growth factor provided by the Casamino Acids but not by the yeast extract must have been stopping growth. However, most of the amino acids must have been synthesized from glutamate; Jebb & Tomlinson (1957) grew small quantities of the organism in serial culture in a medium containing glutamic acid and cystine as the only amino acids.

Proom (1955) found that in simple mixtures of amino acids glutamate could be replaced by succinate or α-ketoglutarate. These compounds are presumably formed from glutamate (see below). In the high glutamate medium with a large inoculum (Rowatt, 1957), the quantity of glutamate (*c.* 2·0 mg.) necessary for the synthesis of 1 mg. dry wt. of organisms contained twice as much nitrogen as the bacteria. Glutamate oxidation, the major metabolic activity of *Bordetella pertussis*, must be efficient energetically if a weight of bacteria is formed from only twice its weight of glutamate.

pH requirements

Hornibrook (1939) recommended pH 7·4 and Cohen & Wheeler (1946) pH 7·2–7·3 for growth of *Bordetella pertussis*. The medium was adjusted to these values before autoclaving. Ungar *et al.* (1950) found an optimal pH value of 7·60–7·85, but growth in their medium was very slow and should not be taken as occurring under optimal conditions. Jebb & Tomlinson (1955) used initial pH 7·9 and Proom (1955) pH 7·6. I found (Rowatt, 1957) that the initial growth rate was the same from pH 6·9 to 7·7, although at pH 7·6–7·7 the growth rate became slower earlier than it did at lower pH values. Consequently I used the lowest possible value, namely pH 6·9–7·0, which gave a medium capable of taking up a larger quantity of alkali than when the initial pH was higher. Some of the ammonia in the Casamino Acids was lost during autoclaving, so that to obtain a medium containing 1 % Casamino Acids at pH 6·9–7·0 it was necessary to adjust to pH 7·2 before autoclaving.

It has been stated above that growth of *Bordetella pertussis* from large inocula in a medium with casein hydrolysate (Benger) was proportional to glutamic acid concentration only up to 0·3 % (w/v) (Rowatt, 1957). The yield of organisms was greater at 0·5 and 1·0 % than at 0·3 % (w/v) glutamate, but growth was limited possibly by the pH value of the medium. Media such as these never gave growth above pH 8·3 and usually pH 8·2 was the limiting value, but in the medium without casein hydrolysate growth stopped at pH 7·8 before all the glutamate had been used (Rowatt, unpublished). Casein hydrolysate may contain some of the factor required but not sufficient for crops greater than 2·5 mg. dry wt./ml.

Utilization of other amino acids in a complex medium

Ungar *et al.* (1950) found that aspartic acid, serine, glycine, threonine, alanine and proline as well as glutamic acid disappeared from media during growth of *Bordetella pertussis*. Proom (1955) reported the utilization of cystine serine, glutamic acid, alanine, proline and leucine; aspartic acid, glycine and threonine were not mentioned. He apparently measured these by paper chromatography using butanol + acetic acid as solvent (see Proom & Woiwod, 1949), and I found (Rowatt, 1955) that serine and aspartic acid travel to the same point in chromatograms of media in this solvent. It is possible therefore that aspartic acid was used.

With 1 % Casamino Acids, I (Rowatt, 1955) did not detect utilization of threonine or leucine, and the cysteine spot was not visible with the butanol + acetic acid solvent. With 0·1 % Casamino Acids, the threonine spot was so faint that it was uncertain whether it was used but the leucine spot was weakened. Alanine was the only spot to alter before the glutamate had been used up; in experiments with low concentrations of amino acids alanine was used but later it sometimes increased (Rowatt, unpublished). In these experiments a small inoculum was used.

With a large inoculum the organism grew in a medium containing glutamate, cysteine and yeast extract as the only nitrogen sources, and in this medium the alanine spot generally increased during growth and so did the valine and leucine spots (Rowatt, unpublished). Valine and methionine have the same R_F value in the solvent used and so do leucine and isoleucine. It is possible that only one member of each pair was utilized and with the leucine + isoleucine spot one member may have been utilized and the other synthesized. However, it is equally possible that the same amino acid may be used or synthesized at different times.

Requirements in a defined medium

Hornibrook (1940) grew *Bordetella pertussis* in a defined medium containing, besides starch and salts, glutamate, tyrosine, glycine, proline, histidine, arginine and cystine. Large inocula grew but an inoculum dilution was reached at which yeast extract became necessary for growth; the yeast extract could be replaced by nicotinamide. Nicotinamide and starch are the only factors for the growth of *B. pertussis* about which all workers are agreed. Hornibrook's (1940) defined medium was not optimal because growth in it could be improved by the addition of casein hydrolysate. Proom (1955) tested this medium (without casein hydrolysate) and found that it would support growth for several subcultures but that alanine was necessary for continued subcultivation. Proom also grew *B. pertussis* through many subcultures in a medium (7 AA) containing asparagine, proline, leucine, alanine, glutamic acid, serine, methionine and cystine. Apart from glutamate and cystine only proline is common to the two media. Proom thought that proline, leucine and alanine were either essential for growth or could only be replaced by complex mixtures of amino acids and almost any combination of three or four amino acids would do to supplement these three.

Jebb & Tomlinson (1955), on the other hand, found that although nicotinamide was the only essential growth factor for *Bordetella pertussis* the purines xanthine, hypoxanthine and guanine increased the quantity of growth, and biotin and haemin made growth more rapid. The effect of these factors was first tested in a medium containing charcoal-treated Casamino Acids with an inoculum of 2×10^2 viable organisms/ml. medium, but a similar relationship was found when glutamate (with cystine) replaced the Casamino Acids, although growth was correspondingly slower (Jebb & Tomlinson, 1957). With glutamate + nicotinamide + purines + biotin + haemin, the quantity and rate of growth did not alter through two further subcultures. The constant yield of bacteria shows that they were not growing on material carried over in the inoculum and the constant growth rate excludes selection of less exacting strains. In the medium with only glutamate + nicotinamide growth, although very slow, was maintained through four subcultures without decrease in the yield of bacteria. The yield of bacteria in these two media was only 0·3 mg. dry wt./ml., possibly because these media were inadequately buffered. I have found (Rowatt, unpublished) that in a medium similar to that of Jebb & Tomlinson (1957) containing 0·01 M-glutamate and 0·1 % (w/v) Casamino Acids, 0·3–0·5 ml. 0·01 N-alkali was formed during the growth of 0·3 mg. dry wt. bacteria. In such a medium the pH value was taken from pH 8·2, the value at which growth usually stops (Rowatt, 1957), to pH 7·6, the starting-point of Jebb & Tomlinson's medium, by approximately 0·2 ml. 0·01 N-acid (Rowatt, unpublished). According to these figures 0·3 mg. dry wt. organism/ml. is slightly more than one would expect from Jebb & Tomlinson's (1957) medium.

The figures of Roberts *et al.* (1955) for *Escherichia coli* show that 0·3 mg. dry wt. bacteria contain *c.* 10 μg. of each of the majority of amino acids. If we assume that these figures hold for *Bordetella pertussis* it follows that, to obtain 0·3 mg. dry wt. bacteria/ml., amino acids which this organism cannot synthesize would have to be added at a concentration of approximately 10 μg./ml. Jebb & Tomlinson's (1957) medium contains 1·5 mg. starch and 1·46 mg. glutamic acid/ml., and in order to add 10 μg. of any other amino acid these compounds would have to contain 1 % of it as contaminant. Since 24 μg. cystine/ml. was added, this amino acid would have to contain 42 % of impurity. These facts make it seem unlikely that any other amino acid is required. However, as the cysteine and glutamic acid used were the natural L-isomers, traces of other growth factors might have been added with them. Biotin and *p*-aminobenzoic acid, for example, are notoriously difficult to remove from natural products.

Micro-organisms which cannot synthesize a given growth factor can sometimes grow in the absence of the factor provided other compounds (which may normally be formed through the mediation of the given factor) are added to the medium. *Lactobacillus casei*, for example, can be grown without added folic acid provided thymine and purine bases are added (Snell, 1951). It is possible that such an effect is responsible for the different results of Proom (1955) and of Jebb & Tomlinson (1955, 1957). Proom also used some natural amino acids, which may have been contaminated with growth factors. It is difficult to assess the value of Proom's medium because he gives no details of time of incubation or yield of bacteria.

Dawson *et al.* (1951) using solid media for growth of *Bordetella pertussis* found that serine inhibited growth, and Jebb & Tomlinson (1955) found an antagonistic effect of the amino acid in liquid media containing a mixture of amino acids. In their experiments, omission of glycine inhibited growth almost completely, but the yield of organisms was restored to two-thirds of that in complete medium by the omission of serine, proline, and methionine as well as glycine. Omission of leucine or isoleucine decreased growth to three-quarters and omission of these two amino acids together to one-quarter of that in the complete medium; omission of valine + leucine + isoleucine restored growth slightly but not to that in the full medium. This suggests that leucine or isoleucine improves the growth of the bacterium as well as overcoming an antagonism by valine. Proom's (1955) 7 AA medium contains the 'inhibitory' amino acids serine, proline and methionine, but not glycine which overcomes the inhibition; it contains leucine but not isoleucine or valine which is the inhibitory amino acid of this set. According to Jebb & Tomlinson's (1955) results, omission of glycine should prevent growth, so the results of these workers are again at variance.

Amino acids may be required by bacteria for non-specific functions; for example, buffering the medium (see Mager, Traub & Grossowicz, 1954) or chelating metals such as iron and preventing their precipitation when the medium is heated. These functions become more important as the medium is simplified. Proom's (1955) statement that several combinations of amino acids would do for growth suggests that they are required for such a non-specific purpose. Proom's medium may be deficient in metals because the salts are boiled together at pH 7·6 without amino acids or starch, both of which tend to lessen the phosphate precipitate formed. When I made this medium with 4·5 μg. Fe/ml., the filtrate contained approximately 0·1 μg. Fe/ml. and sometimes even more iron may be precipitated. Jebb & Tomlinson (1955) boiled their medium at pH 7·9 and then filtered. Such media are not defined so far as inorganic ions are concerned; this method of preparation should be avoided for accurate work. The relative deficiency of iron may have been the reason for my lack of success with these two media (see Rowatt, 1957).

Growth factors needed for rapid and profuse growth

The medium of Jebb & Tomlinson (1957) containing starch, glutamate, cystine, nicotinamide and salts may be the simplest medium which supports growth of *Bordetella pertussis*, but growth is slow and the yield of bacteria small. With small yields the organisms may be growing on impurities in the medium, and it is not certain that they will have the full complement of enzymes or antigens possessed by organisms grown in richer media. From a practical point of view a medium giving rapid profuse growth is needed. A medium containing most amino acids and other compounds ready-made would probably support more rapid growth than one in which these have to be synthesized, but some compounds may cause a disproportionate increase in the rate of growth.

One such factor, or group of factors, for growth of *Bordetella pertussis* is present in blood and possibly at a low concentration in Casamino Acids (Rowatt, 1957). In media containing low concentrations of Casamino Acids, or a high concentration of Casamino Acids at a high initial pH value, or with a small inoculum (less than 5×10^2 organisms/ml.), the organisms grew rapidly with a mean generation time (M.G.T.) of 2·3–2·8 hr. After the 40th to 50th hr. the growth rate decreased and logarithmic growth continued at the new rate with an M.G.T. of 3–5 hr. until growth was almost complete. The primary growth rate was maintained in the presence of 1/100 lysed red blood cells, although not with 1/10,000 lysed red blood cells. I did not do viable counts on cultures containing 1/1000 red blood cells, but a given degree of turbidity appeared more quickly with this concentration of blood than without it and it is probable that enough of the factor or factors was present to keep growth at the primary rate for longer. The secondary growth rate appeared before the concentration of glutamate or ammonia was sufficiently altered to be limiting; it occurred at different pH values when the initial pH of the medium was varied, and it was not caused by lack of oxygen because with the conditions used the aeration rate did not limit growth until more than 0·1 mg. dry wt. organism/ml. was present. The factor was not nicotinamide, boitin or haemin because the effect was present in media containing these compounds (Rowatt, unpublished). It could not be a mixture of amino acids or growth factors because these were added in the 1 % Casamino Acids and in the yeast extract. It probably was not present in sufficient concentration in serum because media containing serum generally grew more slowly than those containing red blood cells (Rowatt, 1957) and inocula from CW agar with serum grew more slowly than those from CW agar with blood (see p. 302). It may be the factor which increased growth when large inocula were used (see p. 310), but this has not been adequately tested.

The slower overall growth rate in media with added nicotinamide instead of yeast extract and with only glutamic acid and cysteine as amino acids (Jebb

& Tomlinson, 1957; Rowatt, 1957) may be due to the time taken to synthesize constituents of the organism. However, I found smaller yields in media in which glutamate replaced Casamino Acids and in some media in which nicotinamide replaced yeast extract. This may have been due to a decrease in amount of organisms formed per unit weight of glutamate, but it may have been due to a deficiency of other factors. If, in a medium giving a small crop of bacteria, the yield can be increased by adding more of a component such as buffer or energy source, other deficiencies may become apparent.

To summarize, media for the growth of *Bordetella pertussis* must not contain colloidal sulphur or sulphides, unsaturated fatty acids or substances tentatively identified as peroxides. Starch, nicotinamide and cystine or cysteine are essential for growth and the yield of organisms is proportional to the concentration of glutamate, within wide limits. Alkali is formed during growth and consequently the medium must be adequately buffered. Different authors recommended initial pH values from pH 7·0 to 7·8. Most authors recommend mild aeration of the medium. According to Jebb & Tomlinson (1955, 1957) slow growth can be obtained in a medium containing starch, nicotinamide, cystine and glutamate; growth is quicker when purines, haemin and biotin are also added. Proom (1955) found this medium to be inadequate and obtained growth with eight amino acids with nicotinamide as the only growth factor. For rapid profuse growth, constituents of blood, yeast extract and casein hydrolysate should be added, together with factors added with large unwashed inocula (Rowatt, 1957). Large inocula are less sensitive than small inocula to the inhibitory substances mentioned at the beginning of this paragraph.

THE METABOLISM OF *BORDETELLA PERTUSSIS*

Knowledge of the metabolism of *Bordetella pertussis* is slight. It is an aerobic organism, and Fujita & Kodama (1934) found that a strain growing on blood agar contained cytochromes a, b, c, d_1, and d_2 but they did not test a true phase I strain. Portwood (1946) found that most phase I strains had catalase activity, but 14 out of 62 did not. She also stated that rough strains of *B. pertussis* had little or no catalase activity. The two phase IV strains I have tested both decomposed hydrogen peroxide (Rowatt, unpublished). The suspensions of the strains found by Portwood (1946) to be inactive may have been too old (see, for example, Cole, quoted in Hinshelwood (1946)).

Bordetella pertussis does not oxidize or ferment any of the usual sugars. However, it contains both ribonucleic and deoxyribonucleic acid and some polysaccharide (Overend, Stacey, Webb & Ungar, 1951), and must be able to metabolize the constituents of these compounds sufficiently to incorporate them into the bacterial substance.

The major metabolic activity of *Bordetella pertussis* is the oxidation of amino acids. Glutamate was the most readily oxidized, but serine, alanine, proline and aspartic acid were also attacked. Ammonia and carbon dioxide were formed from serine, alanine and proline in quantities close to those required for complete oxidation (Jebb & Tomlinson, 1951). The quantity of ammonia formed from aspartic acid was less than the theoretical, but the oxygen uptake and CO_2 output were consistent with complete oxidation. Since the aspartic acid oxidizing system was less stable than the others thick suspensions were used which may have contained sufficient keto acid to take up the missing nitrogen. In a medium containing yeast extract, some growth occurred on serine, proline, alanine and aspartic acid, but the crop was only one-third of that with glutamate as the main source of nitrogen (Jebb & Tomlinson, 1951). Ungar *et al.* (1950) reported that glycine and threonine disappeared from the growth medium of *B. pertussis*, but only 30–50 % of the threonine was used; casein contains only 0·4 % glycine compared with 2–6 % of most other amino acids (Tristram, 1946). This finding is therefore quite consistent with that of Jebb & Tomlinson (1951) that threonine and glycine are not oxidized by *B. pertussis*.

When glutamic acid was oxidized the quantities of oxygen used and of ammonia and carbon dioxide formed were less than those required for complete oxidation (Jebb & Tomlinson, 1951). Acetate, which is formed from glutamate by *Haemophilus parainfluenzae* (Klein, 1940), was not found and Jebb & Tomlinson (1951) assumed that the missing nitrogen was built into cell substance. With a nitrogen-containing compound and a large quantity of bacteria this is probably because the cells contain sufficient of the growth factors required; according to Jebb & Tomlinson (1957) only nicotinamide and cystine need be added to glutamate to give a growth medium. Paper chromatography did not show any amino compounds in the suspension medium after glutamate had been oxidized, but either insufficient of each compound was formed or they were not released from the cells into the medium (Rowatt, 1955).

The first step in oxidation of glutamic acid is usually deamination to α-ketoglutaric acid and evidence for this reaction in *Bordetella pertussis* was produced by Jebb & Tomlinson (1951). They incubated washed suspensions with glutamic acid in the presence of arsenious oxide, which partially blocks the oxidation of keto acids, and isolated a compound whose 2:4-dinitrophenylhydrazone had the same R_F value in two solvents as the derivative of authentic α-ketoglutaric acid. α-Ketoglutarate was oxidized by washed suspensions about half as rapidly as glutamate. Succinate was oxidized rapidly to begin with and pyruvate was oxidized slowly but oxaloacetate, fumarate, malate and acetate did not alter the rate of oxygen uptake (Jebb & Tomlinson, 1951). These compounds are usually intermediates in the oxidation of glutamate by the citric acid cycle, but it is possible that they had no effect because they were unable to get to the active centre in the bacteria. Proom (1955) found that succinate and α-ketoglutarate replaced glutamate for growth; citrate was active for a few phase I strains but lactate and pyruvate were inactive. Jebb & Tomlinson (1951) found that lactate was oxidized rapidly at the beginning of incubation but it is possible that this compound, like alanine, is a poor source of energy. Fukumi *et al.* (1953) found that *B. pertussis* oxidized fumarate, malate, succinate, α-ketoglutarate, citrate and acetate; it is not certain that the strains used by them were in phase I.

Bordetella pertussis does not contain urease and washed suspensions do not form detectable quantities of arginine or ornithine from glutamate or of pigment from tyrosine and glutamate (Rowatt, 1955).

VARIANTS, SPECIES AND GENERA RELATED TO *BORDETELLA PERTUSSIS*

Variations in Bordetella pertussis *produced by media*

Three variations in antigenic and morphological characters of *Bordetella pertussis* have been produced by alteration of the growth media. Toomey & Takacs (1936, 1938) changed the normal short rod to a long organism by adapting freshly isolated strains to veal brain agar or chocolate agar at high pH values. In veal brain broth the organisms were filamentous but became bacillary when returned to veal brain agar. The long forms from chocolate agar could be shortened again by subculture on haemoglobin blood agar (BG) at a lower pH value. A similar change to a bacillary organism occurred during subculture in horse blood agar, and on this medium the organisms became avirulent and filamentous, although the same strain grown on sheep or human blood agar remained virulent and was coccobacillary. This work has not been repeated and its significance is not clear.

Lacey (1951) found that growth of *Bordetella pertussis* on a solid medium containing magnesium sulphate ('magnesium organism') instead of sodium chloride ('sodium organism') changed the antigenic structure. The 'magnesium organism' was not agglutinated by antisera against a 'sodium organism' and antisera against the 'magnesium organism' agglutinated the 'sodium organism' at a lower titre than the 'magnesium organism'. The medium contained 33 % (v/v) horse blood and consequently approximately 0·1 % (w/v) sodium/ml. This effect should be borne in mind when any major alteration to the salt content of the medium is contemplated.

Wittler (1951, 1952) obtained L-forms of *Bordetella pertussis* by ageing, chilling, or by growing inocula of the normal organism on BG plates containing 0·75–1·5 % (w/v) glycine. The L-forms were small round bodies staining red with Giemsa, and long rods and large dark red or purple masses. In order to obtain the L-forms on plates it was necessary to add 1·5 % (w/v) glycine: 0·75 % (w/v) glycine did not always give full transformation. When the normal bacillary form was inoculated into mice, small round red forms could be seen in Giemsa stained phagocytes; the change was more complete in immunized than in normal mice.

Degraded strains of Bordetella pertussis

Leslie & Gardner (1931) found that *Bordetella pertussis* could become changed on continued subculture until finally it grew on solid media without blood. Those who have worked with *B. pertussis* will agree that when one is trying to get the organism to grow on digest agar this transformation takes a long time. Strains frequently grow on blood agar and it seems that blood agar in different laboratories and even at different times in the same laboratory varies in nutritive value. However, to make a strain grow on meat digest agar it may be necessary to subcultivate up to 400 times and even then the strain may grow poorly and die out. Standfast (1951) showed that strains of *B. pertussis* may gain one or more phase IV characteristics while retaining some phase I characteristics. Thus, a strain may become ten times less virulent as tested by intranasal challenge and be able to grow on blood agar while the agglutination titre and the ImD_{50} of the protective antigen remain practically unchanged. The same lack of uniformity is found with biochemical characters.

Table 1. *Comparison of characteristics of strains of* Bordetella pertussis

Strain	Simplest growth medium	Agglutination by phase I antiserum	Growth in autoclaved cysteine	Growth with 'peroxide'	Formation of arginine and ornithine from glutamate	Presence of urease
Phase I	BG	++	–	–	–	–
'Pertussis A'	Blood agar	+	Slow	–	–	–
CN 2217	Digest agar	+	Slow	–	–	–
NCTC 364	Digest agar	–	Slow	–	–	–
G 146 E	Digest agar	–	Slow	?+	+	+
G 154 E	Digest agar	–	Slow	?+	+	+

I (Rowatt, unpublished) have studied 5 strains of *Bordetella pertussis* which are degenerate to a greater or lesser degree (Table 1). The smoothest of these, 'Pertussis A', is the strain used by Pollock (1947), which did not grow on digest agar but grew quite well on blood agar and did not have to be sown very thickly for growth. It was agglutinated by phase I antiserum to a lower titre than a true phase I strain. The five strains were all tested for growth in Cohen & Wheeler's medium to see whether they were inhibited by autoclaved cysteine, and in 0·1 % (w/v) Casamino Acids to see whether peroxides were inhibitory. Growth of 'Pertussis A' in liquid media was slowed but not completely inhibited by autoclaved cysteine and the organism did not grow in 0·1 % (w/v) Casamino Acids unless blood was added, i.e. it was inhibited by 'peroxides' (Rowatt, 1957).

The strain CN 2217 (Wellcome Research Laboratories Collection) obtained from Mr H. Proom is a degraded culture of CN 1262, the American 'Challenge' strain, growing on digest agar. The organisms were rather longer and thinner than those of other strains of *Bordetella*. It did not grow very easily on meat digest agar, tending to form blobs of mucoid material where the inoculum was thickest. It was difficult to emulsify and appeared to grow into the surface of the agar so that the topmost layer was taken off with the bacteria. It grew slowly in Cohen & Wheeler's medium autoclaved with cysteine, but quite quickly when filtered cysteine was added after autoclaving the basal medium. It grew very slowly or not at all with 0·1 % (w/v) Casamino Acids but at the normal rate when blood was added (Rowatt, 1957). The amino acids used during growth were the same as those used by phase I strains. This strain was agglutinated to a low titre by antisera against phase I strains.

The original phase IV strain of Leslie & Gardner (1931) NCTC 364, was isolated before 1920 (Dr S. T. Cowan, private communication) and presumably was subcultivated more or less regularly from that date. It grew on digest agar, but again the growth was rather mucoid and not very profuse, although better than that of strain CN 2217. This strain was not agglutinated by antisera to phase I strains. It grew slowly in the presence of autoclaved cysteine but not with 0·1 % (w/v) Casamino Acids.

The strains G154E and G146E grew profusely on digest agar, giving an easily emulsified growth on the plates and odour of diacetyl, and they grew from small inocula unlike strains NCTC 364 and CN 2217. The strains G154E and G146E grew on digest agar shortly after isolation from cases of whooping cough and were agglutinated to a titre of 1/500 by an antiserum against *Bordetella parapertussis* (Dr J. Ungar, private communication). However, they did not form any pigment on digest agar or in liquid media and were not typical *B. parapertussis*. At a later stage they were not agglutinated by antisera to *B. parapertussis* or to phase I *B. pertussis* or to the other phase IV strains (Rowatt, unpublished). They agglutinated to titre with antisera to each other. These strains grew in the presence of autoclaved cysteine, but growth in 0·1 % (w/v) Casamino Acids was very much slower and was not improved by blood (Rowatt, 1957). This suggests that ‘peroxide’ was not the inhibitory factor in this case. The quantity of organisms formed was very small and it seemed that a growth factor was lacking. Strain G146E, indeed, grew very little even in 1 % (w/v) Casamino Acids and in many experiments in liquid media it did not produce visible turbidity for 10–14 days. Of the amino acids available during growth these two strains usually used only glutamate. Strain G146E used very little of this but strain G154E occasionally used glutamate and alanine. It may be remembered that the other amino acids were not used by the phase I organisms until all the glutamate had disappeared and it is possible that these two strains do not grow vigorously enough to use all the glutamate and consequently do not start to use the other amino acids. They did not ferment the common sugars.

Formation of ornithine and arginine from glutamate

G146E and G154E, when grown on digest agar, oxidized glutamate readily and formed small quantities of arginine and ornithine, as well as CO_2 and ammonia (Baddiley, Rowatt & Standfast, 1952). The ratio of glutamate-N to ammonia was only 0·65 compared with 0·76 obtained with phase I strains (Rowatt, 1955). The other phase IV strains which we have tested did not form enough arginine and ornithine to show on chromatograms of untreated supernatant culture fluid, although they could sometimes be seen when this was concentrated. Both these G strains contained an active urease.

These strains, G146E and G154E, thus seemed to be more closely related to *Bordetella parapertussis* except in pigment formation from tyrosine and in agglutininogens. The other phase IV strains did not differ very much from phase I strains except in the important respect that they grew on agar without blood. All five were different and seem to support the idea of a gradual change from rough to smooth rather than the four steps suggested by Leslie & Gardner (1931).

Relationship to other species

Bordetella parapertussis and *B. bronchiseptica* each resemble *B. pertussis* in using glutamate for growth and needing nicotinamide (Proom, 1955). They are also unable to use carbohydrates, differing in this from *Haemophilus influenzae. B. parapertussis* and *B. bronchiseptica* are not inhibited by colloidal copper sulphide (Proom, 1955) or by 'peroxide' (Rowatt, 1957). The antigens of the Bordetella organisms are related to each other (Eldering & Kendrick, 1938; Kendrick, Nadolski, Eldering & Baker, 1953) and they are not related to those of the Haemophilus group. *B. parapertussis* and *B. bronchiseptica* oxidize glutamate to CO_2 and ammonia and again less ammonia is formed than should be from the glutamate used. *B. parapertussis* forms less ammonia than *B. bronchiseptica* and some of the missing nitrogen appears as arginine and ornithine (Rowatt, 1955). Both contain urease (Moreno-López, 1952). *B. parapertussis* differs from *B. bronchiseptica* and *B. pertussis* in forming a brown pigment during growth (Eldering & Kendrick, 1938). Ensminger (1953) found that the pigment was formed from tyrosine. Washed suspensions formed a pink pigment from tyrosine in the presence of glutamate at pH 7·4 and the pink pigment turned brown on prolonged incubation (Rowatt, 1955). Bradford & Slavin (1937) found catalase in *B. parapertussis* and *B. bronchiseptica* but not in *B. pertussis.* Later work has shown that the last organism generally and possibly always contains catalase (see above).

Each of these organisms grows on digest agar, *Bordetella bronchiseptica* rapidly and profusely and *B. parapertussis* less readily. *B. bronchiseptica* will grow from a small inoculum but it is necessary to use quite a large inoculum of *B. parapertussis* or it dies out (Rowatt, unpublished). Proom (1955) showed that *Bordetella bronchiseptica* is the least exacting of the three species, growing on an inorganic salts medium + nicotinic acid + glutamate + proline + leucine, although some strains also required cystine + methionine. All strains of *B. parapertussis* needed cystine and methionine. *B. pertussis* did not grow in this medium, although it grew when asparagine + alanine + serine + starch were added. As stated above, there is some controversy about adequacy of this medium for *B. pertussis. B. bronchiseptica* grew most rapidly of the three in the Cohen & Wheeler type medium and this medium seemed to contain all needed growth factors. *B. parapertussis* grew more slowly and the growth rate decreased when growth was just visible (Rowatt, 1955). With small quantities of blood turbidity of *B. parapertussis* became visible more quickly (Rowatt, 1957) and it is probable that *B. parapertussis* needs the factor present in blood which is required by *B. pertussis* for rapid sustained growth (see p. 315). This requirement shows up more with *B. parapertussis*, possibly because inocula of this organism were grown on digest agar without blood while those of *B. pertussis* were grown on a medium containing 30 % (v/v) blood. The liquid medium used may contain traces of the factor. In the Cohen & Wheeler type medium *B. bronchiseptica* gives a greater turbidity for the same quantity of glutamate than the other two organisms (Rowatt, 1955), but it is possible that non-nitrogenous substances are also being used (Proom, 1955) and the efficiency is not really greater.

Relation of the Bordetella group to other genera

The three organisms of the Bordetella group are thus closely related. They have recently been separated from the Haemophilus group (Moreno-López, 1952; Proom, 1955; Pittman, 1955) on nutritional and antigenic grounds. On antigenic grounds (the common O antigen, toxin and site of infection) the division is valid. However, little is known of the metabolism of these organisms.

The pertussis organism was originally included in the genus *Haemophilus* because it was 'blood-loving'. The type organism of this genus, *H. influenzae*, required a heat-stable factor from blood (X-factor) and a heat-labile factor obtained either from blood or vegetable material (V-factor), and other strains in the group required one or both of these factors. Fildes (1923) showed that two strains of the pertussis organism would grow in peptone water without either X- or V-factor, and he wanted to exclude it from the genus. Certainly one, and probably both, of the strains which he used had been isolated 3 years previously (Dr S. T. Cowan, private communication) and were not in phase I. Hornibrook (1940) showed that *Bordetella pertussis* required nicotinamide which is a constituent of coenzyme I (DPN), and Jebb & Tomlinson (1955) found an improvement of its growth in the presence of haemin. *Haemophilus canis* requires haemin for growth but not DPN, and it is possible, although it has never been proved, that this organism needs nicotinamide for growth. Classically *H. parainfluenzae* does not need haemin for growth, but small inocula of this organism did not grow unless haemin or substances such as ferrous sulphate or manganese dioxide were added (Gilder & Granick, 1948). These substances destroy peroxide and some of them exerted a sparing effect on haemin in the growth of *H. influenzae* (Gilder & Granick, 1948). *Bordetella bronchiseptica* and *B. parapertussis* do not require haemin but both need nicotinamide (Proom, 1955), and thus from a nutritional point of view there is a case for including them in the same genus as the Haemophilus organisms.

The requirement for haemin under highly aerobic conditions is shared by *Pasteurella pestis* (Herbert, 1949), but this organism does not need nicotinamide for growth, and it needs a more complex amino acid mixture than the Bordetella group. The Brucella group use carbohydrates (McCullough & Beal, 1951; Roessler, Sanders, Dulberg & Brewer, 1952) and need thiamine, pantothenate and biotin for growth as well as nicotinamide (Koser, Breslove & Dorfman, 1941). *Brucella abortus* and *B. melitensis* resemble *Bordetella parapertussis* and the two phase IV strains G154E and G146E in forming arginine and ornithine from glutamate (Cameron, Holm & Meyer, 1952), and *Brucella abortus* and, to a lesser extent, *B. melitensis* resemble *Bordetella pertussis* in being inhibited by autoclaved cysteine (Schuhardt, Rode & Oglesby, 1949).

There are thus similarities and differences between all four groups and until the metabolic characteristics are more fully understood they should not be made the basis of classification.

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REFERENCES

Baddiley, J., Rowatt, E. & Standfast, A. F. B. (1952). The metabolism of glutamate by some rough strains of *Haemophilus pertussis*. *Proc. Int. Congr. Biochem.* p. 75.

Bailey, J. H. (1933). A medium for the isolation of *Bacillus pertussis*. *J. infect. Dis.* **52**, 94.

Barksdale, I. S., Mullenix, G. K. & Fuster, L. B. (1941). Further studies on a simplified cough-plate method for the early diagnosis of whooping cough. *Sth. Med. Surg.* **103**, 176.

Barksdale, I. S. & Simpson, F. P. (1934). A simplified cough-plate method for the early diagnosis of whooping cough. *Sth. med. J., Nashville*, **27**, 943.

Barry, V. C., Conalty, M. L., Denneny, J. M. & Winder, F. (1956). Peroxide formation in bacteriological media. *Nature, Lond.* **178**, 596.

Bordet, J. & Gengou, O. (1906). Le microbe de la coqueluche. *Ann. Inst. Pasteur*, **20**, 731.

Bordet, J. & Sleeswyk (1910). Sérodiagnostic et variabilité des microbes suivant le milieu de culture. *Ann. Inst. Pasteur*, **24**, 476.

Bradford, W. L. & Slavin, B. (1937). An organism resembling *Haemophilus pertussis*. With special reference to colour changes produced by its growth upon certain media. *Amer. J. publ. Hlth*, **27**, 1277.

Cameron, H. S., Holm, L. W. & Meyer, M. E. (1952). Comparative metabolic studies on the genus Brucella. 1. Evidence of a urea cycle from glutamic acid metabolism. *J. Bact.* **64**, 709.

Cohen, S. M. & Wheeler, M. W. (1946). Pertussis vaccine prepared with phase-I cultures grown in fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

Cruickshank, J. C. & Freeman, G. G. (1937). Immunising fractions isolated from *Haemophilus pertussis*. *Lancet*, ii, 567.

Davis, B. D. & Dubos, R. J. (1946). Interaction of serum albumin, free and esterified oleic acid and lipase in relation to cultivation of the tubercle bacillus. *Arch. Biochem.* **11**, 201.

Dawson, B., Farnworth, E. H., McLeod, J. W. & Nicholson, D. E. (1951). Observations on the value of the Bordet-Gengou medium for the cultivation of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 408.

Debré, R., Marie, J. & Pretet, H. (1928). Les races de bacilles de Bordet-Gengou isolées à Paris. *C.R. Soc. Biol., Paris*, **98**, 761.

Eldering, G. & Kendrick, P. (1938). *Bacillus para-pertussis*: a species resembling both *Bacillus pertussis* and *Bacillus bronchisepticus* but identical with neither. *J. Bact.* **35**, 561.

Ensminger, P. W. (1953). Pigment production by *Haemophilus parapertussis*. *J. Bact.* **65**, 509.

Ensminger, P. W., Culbertson, C. G. & Powell, H. M. (1953). Antigenic *Hemophilus pertussis* vaccines grown on charcoal agar. *J. infect. Dis.* **93**, 266.

Farrell, L. & Taylor, E. M. (1945). Notes on the production of phase I pertussis vaccine in fluid medium. *Canad. J. publ. Hlth*, **36**, 326.

FILDES, P. (1923). The classification of haemoglobinophilic bacteria, based upon their relation to blood-pigment and to the 'vitamine' factor. *Brit. J. exp. Path.* **4**, 265.

FISHER, S. (1948). The behaviour of *Haemophilus pertussis* in casein hydrolysate broth. *Aust. J. exp. Biol. med. Sci.* **26**, 299.

FUJITA, A. & KODAMA, T. (1934). Untersuchungen über Atmung und Gärung pathogener Bakterien. III. Mitteilung. Über Cytochrom und das sauerstoffübertragende Ferment, sowie die Atmungshemmung der pathogenen Bakterien durch CO und HCN. *Biochem. Z.* **273**, 186.

FUKUMI, H., SAYAMA, E., TOMIZAWA, J. & UCHIDA, T. (1953). Nutritional requirements and respiratory pattern of pertussis-parapertussis-bronchisepticus group of microorganisms. *Jap. J. med. Sci. Biol.* **6**, 587.

GILDER, H. & GRANICK, S. (1948). Studies on the Haemophilus group of organisms. Quantitative aspects of growth on various porphin compounds. *J. gen. Physiol.* **31**, 103.

HERBERT, D. (1949). Studies on the nutrition of *Pasteurella pestis*, and factors affecting the growth of isolated cells on an agar surface. *Brit. J. exp. Path.* **30**, 509.

HINSHELWOOD, C. N. (1946). *The Chemical Kinetics of the Bacterial Cell*, 1st edn., p. 47. Oxford: Clarendon Press.

HORNIBROOK, J. W. (1939). Cultivation of phase I *H. pertussis* in a semi-synthetic liquid medium. *Publ. Hlth Rep., Wash.* **54**, 1847.

HORNIBROOK, J. W. (1940). Nicotinic acid as a growth factor for *H. pertussis*. *Proc. Soc. exp. Biol., N.Y.* **45**, 598.

JEBB, W. H. H. & TOMLINSON, A. H. (1951). The catabolic activity of washed suspensions of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 951.

JEBB, W. H. H. & TOMLINSON, A. H. (1955). The nutritional requirements of *Haemophilus pertussis*. *J. gen. Microbiol.* **13**, 1.

JEBB, W. H. H. & TOMLINSON, A. H. (1957). The minimal amino acid requirements of *Haemophilus pertussis*. *J. gen. Microbiol.* **17**, 59.

KENDRICK, P. L., NADOLSKI, E. B., ELDERING, G. & BAKER, J. (1953). Antigenic relationships of *Haemophilus pertussis*, the parapertussis bacillus and *Brucella bronchiseptica* as shown by cross protection tests in mice. *J. Bact.* **66**, 166.

KLEIN, J. R. (1940). The oxidation of L(−)-aspartic and L(+) glutamic acids by *Hemophilus parainfluenzae*. *J. biol. Chem.* **134**, 43.

KLIMENKO, W. N. (1908–09). Die Ätiologie des Keuchhustens. *Zbl. Bakt.* (Abt. 1 Orig.), **48**, 64.

KOSER, S. A., BRESLOVE, B. B. & DORFMAN, A. (1941). Accessory growth factor requirements of some representatives of the Brucella group. *J. infect. Dis.* **69**, 114.

KRISTENSEN, M. (1927). Recherches sérologiques sur le bacille de la coqueluche. *C.R. Soc. Biol., Paris*, **96**, 355.

KRUMWIEDE, C., MISHULOW, L. & OLDENBUSCH, C. (1923). The existence of more than one immunologic type of *B. pertussis*. *J. infect. Dis.* **32**, 22.

LACEY, B. W. (1951). Antigenic modulation of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, xxi.

LACEY, B. W. (1954). A new selective medium for *Haemophilus pertussis* containing a diamidine, sodium fluoride and penicillin. *J. Hyg., Camb.* **52**, 273.

LAWSON, G. McL. (1939). Immunity studies in pertussis. *Amer. J. Hyg.* (B), **29**, 119.

LESLIE, P. H. & GARDNER, A. D. (1931). The phases of *Haemophilus pertussis*. *J. Hyg., Camb.* **31**, 423.

McCULLOUGH, N. B. & BEAL, G. A. (1951). Growth and manometric studies on carbohydrate utilization of Brucella. *J. infect. Dis.* **89**, 266.

MACKIE, T. J. & McCARTNEY, J. E. (1953). *Handbook of Practical Bacteriology*, 9th edn., p. 189. Edinburgh: E. and S. Livingstone Ltd.

MADSEN, T. (1925). Whooping cough. Its bacteriology, diagnosis, prevention and treatment. *Boston med. & surg. J.* **192**, 50.

MAGER, J., TRAUB, A. & GROSSOWICZ, N. (1954). Cultivation of *Pasteurella tularensis* in chemically defined media: effect of buffers and spermine. *Nature, Lond.* **174**, 747.

MAZLOUM, H. A. & ROWLEY, D. (1955). The growth requirements of *Haemophilus pertussis* on solid media. *J. Path. Bact.* **70**, 439.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

MISHULOW, L., SHARPE, L. S. & COHEN, L. L. (1953). Beef-heart charcoal agar for the preparation of pertussis vaccines. *Amer. J. publ. Hlth*, **43**, 1466.

MORENO-LÓPEZ, M. (1952). El genero Bordetella. *Microbiol. esp.* **5**, 177.

OVEREND, W. G., STACEY, M., WEBB, M. & UNGAR, J. (1951). The isolation of deoxyribonucleic acid from virulent and avirulent strains of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 268.

PITTMAN, M. (1955). The genus *Bordetella* Moreno-López, 1952. In Breed, R. S. *et al.* (1955). Symposium on Taxonomy. *Bact. Rev.* **19**, 273.

POLLOCK, M. R. (1947). The growth of *H. pertussis* on media without blood. *Brit. J. exp. Path.* **28**, 295.

POLLOCK, M. R. (1948). Unsaturated fatty acids in cotton wool plugs. *Nature, Lond.* **161**, 853.

POLLOCK, M. R. (1949). The effect of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. *Symp. Soc. exp. Biol.* **3**, 193.

POLLOCK, M. R., HOWARD, G. A. & BOUGHTON, B. W. (1949). Long-chain unsaturated fatty acids as essential bacterial growth factors. Substances able to replace oleic acid for the growth of *Corynebacterium* '*Q*' with a note on a possible method for their microbiological assay. *Biochem. J.* **45**, 417.

PORTWOOD, L. M. (1946). Catalase activity of *Hemophilus pertussis*. *J. Bact.* **51**, 265.

POVITSKY, O. R. (1923). Improved methods for the isolation and later cultivation of *B. pertussis*. *J. infect. Dis.* **32**, 8.

POWELL, H. M., CULBERTSON, C. G. & ENSMINGER, P. W. (1951). Charcoal agar culture medium for preparing *Haemophilus pertussis* vaccine. *Publ. Hlth Rep., Wash.* **66**, 346.

PROOM, H. (1955). The minimal nutritional requirements of organisms of the genus *Bordetella* López. *J. gen. Microbiol.* **12**, 63.

PROOM, H. & WOIWOD, A. J. (1949). The examination, by partition paper chromatography, of the nitrogen metabolism of bacteria. *J. gen. Microbiol.* **3**, 319.

ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T. & BRITTEN, R. J. (1955). Studies of biosynthesis in *Escherichia coli*. *Publ. Carneg. Instn*, **607**, Washington D.C.

ROESSLER, W. G., SANDERS, T. H., DULBERG, J. & BREWER, C. R. (1952). Anaerobic glycolysis by enzyme preparations of *Brucella suis*. *J. biol. Chem.* **194**, 207.

ROWATT, E. (1955). Amino acid metabolism in the genus *Bordetella*. *J. gen. Microbiol.* **13**, 552.

ROWATT, E. (1957). Some factors affecting the growth of *Bordetella pertussis*. *J. gen. Microbiol.* **17**, 279.

SCHUHARDT, V. T., RODE, L. J. & OGLESBY, G. (1949). The toxicity of certain amino acids for brucellae. *J. Bact.* **58**, 665.

SCHUHARDT, V. T., RODE, L. J., OGLESBY, G. & LANKFORD, C. E. (1950). The development of peptone toxicity for brucellae with aging and the correlation of this toxicity with the probable oxidation of cystine. *J. Bact.* **60**, 655.

SCHUHARDT, V. T., RODE, L. J., OGLESBY, G. & LANKFORD, C. E. (1952). Toxicity of elemental sulphur for brucellae. *J. Bact.* **63**, 123.

SILVERTHORNE, N. & CAMERON, C. (1942). Whooping cough. IV. Beef liver extract blood agar media for growth of *H. pertussis*. *J. Pediat.* **20**, 16.

SNELL, E. E. (1951). In *Bacterial Physiology*, p. 231. Ed. Werkman, C. H. & Wilson, P. W. New York: Academic Press Inc.

STANDFAST, A. F. B. (1951). The phase I of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 531.

TOOMEY, J. A. & MCCLELLAND, J. E. (1933–34). Pertussis toxin or antigen. *Proc. Soc. exp. Biol., N.Y.* **31**, 34.

TOOMEY, J. A. & TAKACS, W. S. (1936). Changes in morphology of *Hemophilus pertussis* grown under varying conditions and on different mediums. *J. Bact.* **31**, 44.

TOOMEY, J. A. & TAKACS, W. S. (1938). The effect of various blood mediums on the growth characteristics of *H. pertussis*. *J. infect. Dis.* **62**, 297.

TRISTRAM, G. R. (1946). Observations upon the application of partition chromatography to the determination of the monoamino-acids in proteins. *Biochem. J.* **40**, 721.

UNGAR, J., JAMES, A. M., MUGGLETON, P. W., PEGLER, H. F. & TOMICH, E. G. (1950). The cultivation of *Haemophilus pertussis* in partially defined liquid media. *J. gen. Microbiol.* **4**, 345.

VERWEY, W. F., THIELE, E. H., SAGE, D. N. & SCHUCHARDT, L. F. (1949). A simplified liquid culture medium for the growth of *Hemophilus pertussis*. *J. Bact.* **58**, 127.

WEICHSEL, M., RUBIN, H. J., COHEN, P. & LAPIN, J. H. (1940). Intracutaneous tests with pertussis 'toxin' and complement fixation tests in whooping cough. *Amer. J. Dis. Child.* **60**, 862.

WILSON, R. J. (1945). The production of phase I Pertussis vaccine in casein hydrolysate broth. *Canad. J. Publ. Hlth*, **36**, 321.

WITTLER, R. G. (1951). The L-form of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, 1024.

WITTLER, R. G. (1952). The L-form of *Haemophilus pertussis* in the mouse. *J. gen. Microbiol.* **6**, 311.

WOIWOD, A. J. (1954). The inhibition of bacterial growth by colloidal heavy metal sulphides and by colloidal sulphur. *J. gen. Microbiol.* **10**, 509.

W.H.O. Report (1953). Diphtheria and pertussis vaccination. Report of a conference of heads of laboratories producing diphtheria and pertussis vaccines. *World Hlth Org. techn. Rep. Ser.* no. 61.

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Cooper, P. D. (1957). *J. gen. Microbiol.* **17**, 327–334

Some Characteristics of Vesicular Stomatitis Virus Growth-Curves in Tissue Culture

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SUMMARY: Vesicular stomatitis virus was released exponentially and fairly reproducibly after infecting chick embryo cell monolayers. This system appeared suitable for chemical studies, which are to be presented separately; the present paper is concerned with growth-curve methods and a discussion of the system. When the average multiplicity of adsorption was 6 plaque-forming units/cell the latent period was 1–2 hr., and growth appeared to follow a one-step cycle. At least 62–75 % of the cells were capable of releasing virus.

This paper is intended to show that vesicular stomatitis (VS) virus growing in chick embryo-cell monolayers is a suitable system for quantitative study. It describes the conditions used in such studies, the results of which are to be presented separately (Cooper, 1957*a*, *b*).

Intracellular chemical changes during infection should give information on virus growth, but a suitable system needs the following properties: (*a*) virus should be assayed by particle count methods and should be released into a fluid medium and not extracted in unknown yield from whole tissue; (*b*) one-step growth conditions must apply, so that changes take place in all cells together as much as possible (the most informative changes occur during the latent or exponential release periods which must be identifiable); (*c*) nearly all cells must be infected at zero time, so that pathological changes are not masked by metabolism of healthy cells; (*d*) samples must be representative and reproducible; (*e*) cells should produce virus at an optimal rate, so that changes are maximal. These criteria are discussed below in relation to the system used. It is unfortunate that technical difficulties have hindered interpretation of many experiments with animal viruses (Bauer, 1952), because at least one of these properties was missing.

METHODS

General type of experiment. Most experiments involved harvesting one virus-infected and one control monolayer of embryo cells and the entire supernatant fluids after varying times during incubation at 37°. A group of monolayers all from the same batch were infected nearly simultaneously as described below, and a growth curve was obtained by plotting the total plaque-forming units (pfu) in the completely removed supernatant fluids against the time of harvesting. Thus six points on a growth curve would require six monolayers; successive samples from the same plate were not used. The method for harvesting the cells is given with the chemical methods (Cooper, 1957*a*).

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Virus growth and phosphorus metabolism should be similar in cells prepared identically from chick embryos of the same age, and the state of any one monolayer and supernatant at a given time should represent that of all others from the same batch when treated identically. That these assumptions are justified is supported by: (*a*) the reproducibility of the growth curves discussed below; (*b*) the fairly smooth and reproducible nature of the ^{32}P uptake curves (Cooper, 1957*a*) and the low standard deviations of the total ^{32}P taken up by two sets of 10 identical monolayers (about 5 % of the mean uptakes).

Virus stocks. The virus used was a New Jersey (Missouri) strain of vesicular stomatitis virus kindly provided by Dr S. Madin, which had been shown to make good plaques on chick cells by Sellers (1955 and personal communication). All stocks were derived by diluted inoculum passage from a master culture, which was the 10 hr. supernatant fluid (5 ml.) from a chick embryo monolayer infected with a suspension of a single well-separated plaque.

The presence of a transmissible interfering component ('incomplete virus'?) in some vesicular stomatitis virus stocks is to be reported elsewhere. All experiments described here and by Cooper (1957*a*, *b*) used stocks which are regarded as containing very little of this component, as they had high titres and were made by not more than three diluted passages from the original presumed single particle. Virus yields (pfu/cell) during experiments were high.

Stocks were kept at −20° for not more than 3 months; the titre was generally 2×10^9 pfu/ml., but some inactivation was detected on storage. Each cycle of freezing and thawing in Earle's saline + 4 % (v/v) serum resulted in an inactivation of about 15 % of the plaque-forming units.

Virus assay. The plaque method used was that described by Rubin, Baluda & Hotchin (1955) for Western Equine Encephalomyelitis virus and cells from 10-day chick embryos, except that embryo extract in the agar overlay was replaced by 5 mg./ml. lactalbumin hydrolysate + 1 mg./ml. yeast extract + 1 mg./ml. crystalline bovine plasma albumin (Dr J. S. Youngner, personal communication). The curves presented are relative assays not corrected for plating efficiency, and amounts of virus are described in terms of plaque-forming units in the assay system used. Growth and storage took place entirely in darkness, and during virus assays virus was in serum-free media for less than 5 min. under exclusively artificial (fluorescent strip) lighting, so that light inactivation (Skinner & Bradish, 1954) was negligible. One plaque results from a single 'infective centre' (Franklin, personal communication); the 'infective centre' may be a clump of virus particles but virus preparations gave single-hit X-ray and UV inactivation curves and were therefore presumed to be monodisperse, so that one plaque results from one infective virus particle. The average multiplicity of infection was determined from the number of pfu adsorbed by the monolayer and the number of cells present in the monolayer. The number of cells was found by haemocytometer counts of a trypsin re-suspension. The number of pfu adsorbed to individual cells was assumed to follow a Poisson distribution. Whether all the particles adsorbed to a cell or only a fraction thereof participate in viral reproduction within that cell is at present unknown.

Media. Earle's saline (1943) containing 4% (v/v) horse or more often ox serum (ES/Ox) was added to infected or control monolayers for the period of virus release; no difference was found between sera provided that antiviral activity was absent. Serum was included for its small stimulation of the rate of ^{32}P uptake and better effect on appearance of cells, particularly after 20 hr. Virus was diluted for assay in phosphate buffer saline (PBS, Dulbecco & Vogt, 1954*a*).

Preparation of monolayers of chick embryo cells. Monolayers were prepared for growth-curves and for routine plaque-assays by the same method. This was either the pulping procedure described by Rubin *et al.* (1955), or else the embryos were coarsely minced and digested by gentle agitation in trypsin (Melnick, 1955). The latter method gives three to four times as many cells as the former and the performance of the monolayers appears to be identical. Monolayers were always used for growth-curves (and usually for assays) after 20 hr. incubation when cells had spread to form a continuous sheet. The ^{32}P uptake continued at a constant rate for the first 2 days but had nearly ceased by the third day.

Method employed for one-step growth-curves. To infect the cells, medium was removed from 20 hr.-monolayers which were then washed once (or three times when containing ^{32}P) with 5 ml. ES/Ox. After washing, 0·5 ml. of ES/Ox containing virus at known titre (or without virus for controls) was added, and allowed to spread over the whole surface (2–3 min.), when the plates were incubated at 37° in a humid atmosphere containing approximately 5% CO_2 for 30 min. Addition of 4·5 ml. of warm ES/Ox was followed by washing in some experiments, but where washing is not mentioned the medium was allowed to remain on the plates at 37° until entirely removed at different times and frozen pending virus assay. Unless otherwise mentioned, the inoculum contained 7·5 pfu for each cell in the monolayer, and assay of the medium shortly after making up to 5 ml. showed that adsorption was 60–90% complete (average 80%) giving a final average multiplicity of adsorption of 5–6. Little further VS virus was adsorbed by 10 cm. Petri dish monolayers after 30 min. with 0·5 ml. of inoculum, and an average multiplicity of 1 was probably reached 10–15 min. after its addition. The time of first addition of virus was regarded as zero, so that the latent periods include the period required for the virus to come into contact with the cells.

RESULTS

Establishment of the time required for a presumed one-step growth-curve

Fig. 1 shows the virus release-time relationships obtained from a number of experiments using the growth-curve method described above. Duplicates agreed well, and fairly smooth curves were obtained. The latent period is defined throughout the present work as the average time required for each cell to release one pfu; this is regarded as the least ambiguous way of defining the rate of development of a system in which no sharply delineated 'burst' occurs. The latent period is determined when necessary by extrapolating the

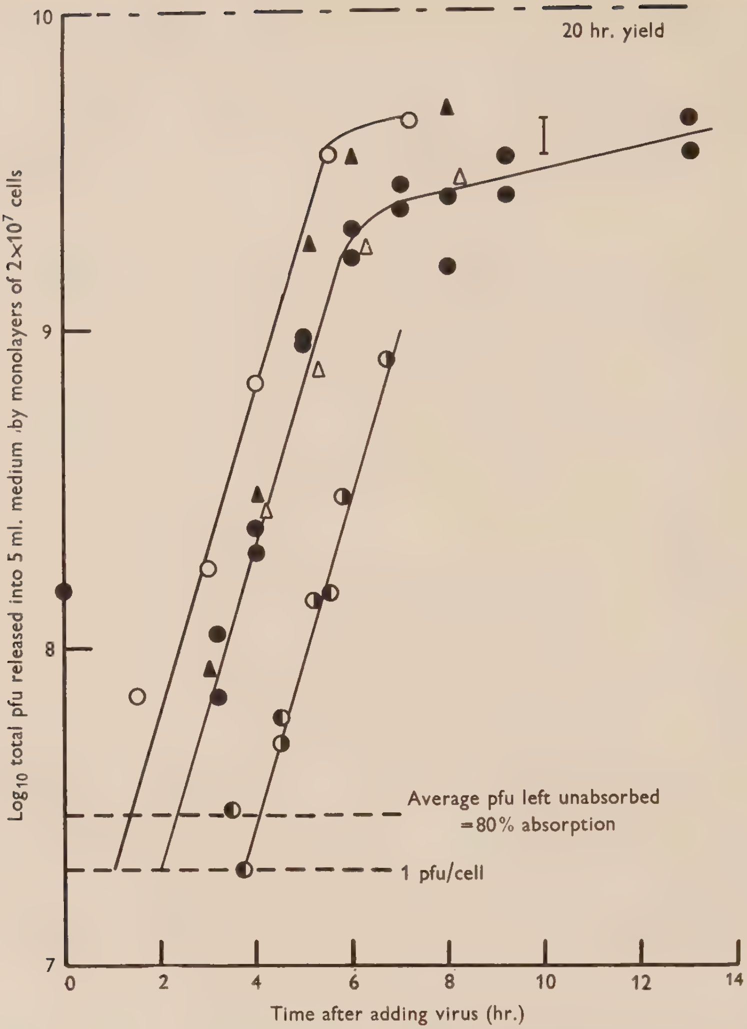

Fig. 1. Total number of plaque-forming units released by chick embryo cell monolayers (2×10^7 cells) infected to an average multiplicity of adsorption of 6 with vesicular stomatitis virus. All points of each curve were obtained with the same batch of plates, but except where mentioned the complete curves were made on separate occasions with different batches of plates. Two experiments (○—○, washed once; ●—●, washed three times) are given to show the extreme range in latent period encountered among twelve experiments; in all the experiments virus release increased exponentially at similar rates. Variations between duplicates are shown by (●—●); (▲—▲) was washed once and (△—△) was washed three times with medium before infection, but were otherwise identical and from the same batch of plates. Two curves (◑—◑) and (◐—◐) were infected with multiplicity 1–2 (uncorrected for cells theoretically uninfected) but otherwise were identical with those of multiplicity$=6$. The bar at 10 hr. indicates the range of the yields from seven different multiplicities of adsorption varying from 0·5 to 23. The point on the ordinate represents the virus added as inoculum for multiplicity$=6$, and the middle broken horizontal line the average amount of virus left unadsorbed after 30 min.; the lowest broken line indicates the level of termination of the latent period, and the uppermost broken line the average 20 hr. yield from once-washed monolayers.

exponential rise in a logarithmic plot back to 1 pfu/cell. The average latent period was 1·5 hr. at a multiplicity of 6; all latent periods at this multiplicity fell within the range of 1·0–2·0 hr. Exponential release of 50 % of the final yield occurred up to 6 hr., and the remainder was released more slowly for some hours further. Although the latent periods varied somewhat between experiments, the rates of virus release were closely similar, and except as described the final yields were also similar; the threefold washing of the plates before infection required by some experiments gave a considerably lower yield than one wash. The small differences in latent period may be due to factors (such as small variations between batches of plates) other than differences in washing of the plates, as one experiment with the same batch of plates where the washings differed gave similar latent periods although slightly different rates of release and different final yield.

Proportion of cells supporting viral growth

Adsorption of 12×10^7 pfu by a uniform confluent monolayer of 2×10^7 cells should be sufficient to place virus on 99·7 % of the cells. That most cells with adsorbed virus are capable of supporting growth is regarded as proved by the following observations: (*a*) 40 hr. plaques on chick embryo monolayers show a fairly sharp boundary within which no live cells are visible, so that none of the population is completely resistant; (*b*) as described elsewhere (Cooper, 1955), when cells were removed during the latent period with 0·25 % trypsin from monolayers infected to multiplicity of 6–7, 62–75 % of the cells counted in a haemocytometer were able to produce plaques when mixed in an agar cell suspension containing 2×10^7 non-infected cells/ml. All cells visible were included in the count, even those apparently in poor condition, so that the considerable chance of cell trauma during their treatment means that this figure of 62–75 % is certainly appreciably low. Evidence was presented by Dulbecco & Vogt (1954*a*) to show that cells in a monkey kidney monolayer were equally susceptible to poliovirus.

Effect of multiplicity of adsorption

Fig. 1 shows that the two release curves with multiplicity 1 to 2 had a longer latent period than those with multiplicity 6, while the rate of release and 10 hr. yield were independent of multiplicity. These effects will be discussed more extensively elsewhere. Meanwhile the presentation of these experiments is anticipated in Fig. 2, as the effect of multiplicity of adsorption on latent period has some important inferences for chemical studies.

As cells probably adsorb virus according to a Poisson distribution, the multiplicity of individual cells in an infected monolayer will vary. The effect of multiplicity of adsorption on latent period will mean therefore that at any one time the cells in such a monolayer will be at different stages of their growth cycle. This is a characteristic undesirable for chemical study but one which appears inevitable if one insists that all cells are infected initially. To estimate the extent of the discrepancies, theoretical release curves were constructed for an ideal monolayer randomly infected to an average multiplicity of 6 by applying the observed relationships between multiplicity and latent period, and assuming that cells at different multiplicities release virus at the same exponential rate and to the same yield, even though starting at different times. It is further assumed that all cells of the same multiplicity start together, and the later non-exponential release is ignored. Some illustrative curves are given in Fig. 2. From these curves it can be calculated that 80 % of the cells are less than 0·7 hr. apart in starting or completing their release, 60 % < 0·4 hr. apart and 30 % < 0·1 hr. apart; a negligible proportion of cells complete their release more than 0·6 hr. before the mean time for completion. As samples are

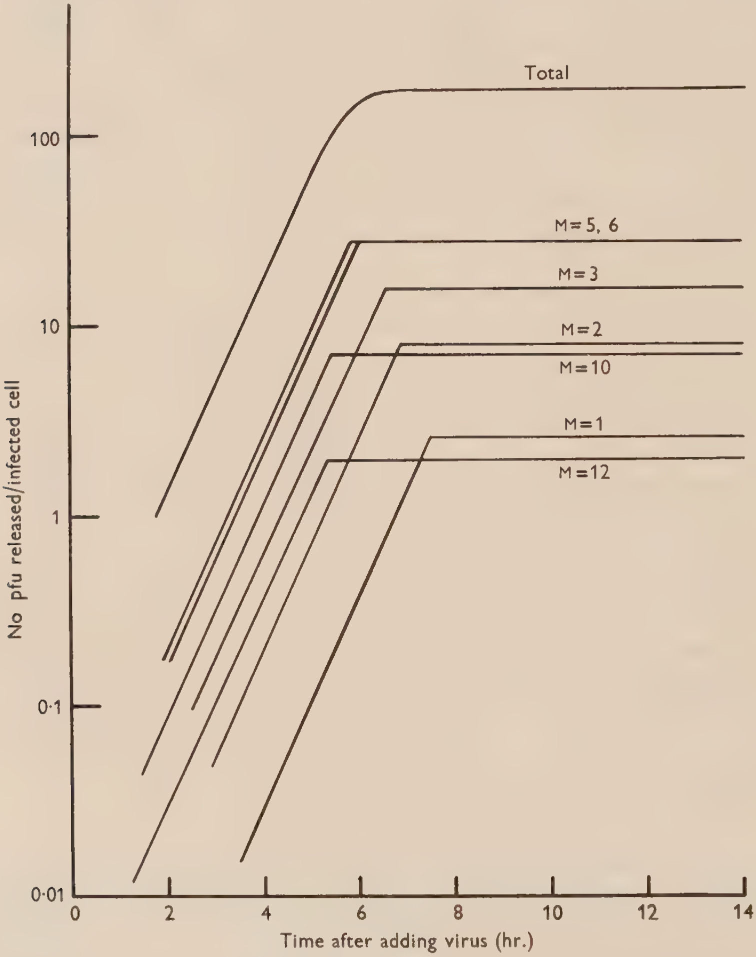

Fig. 2. Theoretical curves attempting to evaluate the differences in virus release to be expected from a Poisson distribution of multiplicity of infection in a monolayer infected to an average multiplicity of 6 (multiplicities higher than 12 are ignored). The commencement of each curve corresponds with release of one pfu for the average cell at that multiplicity, and is calculated from the latent periods of monolayers infected to that multiplicity *as an average*; only seven of the twelve curves are given.

usually taken 1 hr. apart this probably means that virus growth will be sufficiently synchronized for any chemical change due to virus growth to be the major one, and not to be too obscured by healthy metabolism of late-starting cells or lysis of early starters. Nevertheless, the fact that these curves represent only an average would have to be borne in mind in, for example, placing any small change in its correct time sequence with regard to virus growth. The theoretical curves do not, of course, consider cells which are early or late starters for reasons other than multiplicity, and some further spread will be introduced by the non-simultaneous infection of the cells due to the 30 min. adsorption period required.

DISCUSSION

That the experiments described above represent one-step growth cycles is demonstrated by: (*a*) relatively rapid adsorption of sufficient plaque-forming units to infect all cells initially; (*b*) release of virus by most infected cells; (*c*) the release of 50 % of virus within the exponential period, without previous or subsequent steps; (*d*) the similarity of other animal virus growth systems likely to be one-step curves (Dulbecco, 1955); (*e*) the almost simultaneous cessation of ^{32}P uptake by many fractions without concurrent lysis during the exponential period (Cooper, 1957*a*), indicating that most cells are affected at this time.

Continued slow non-exponential release may be a function of delayed cell death, as by 6 hr. less than 25 % of the cells were 'rounded-up' and release of phosphorus from most cellular fractions was very small (Cooper, 1957*a*). Thus non-specific lysis at this stage must also be small. Most cells were rounded and lysis was extensive by 20 hr.

It is a disadvantage of this system that the cell population is not homogeneous and can be seen to contain fibroblast and epithelial type cells. However, fibroblasts are in considerable excess, and a mixture probably does not matter for the present purpose, as McClain & Hackett (1955) showed that latent and release periods of VS virus were closely similar in three epithelial and two fibroblast cultures from five different vertebrate species; of these systems chick cells produced most virus and therefore may show most changes. The intact monolayer was preferred for virus growth to the cell suspension method for this reason, as the latter often gave lower yields per cell (see also Dulbecco & Vogt, 1954*b*).

It thus appears that most of the criteria listed in the introduction as necessary for chemical studies are fulfilled by this system.

I am very grateful to my colleagues at the California Institute for stimulating discussions; in particular to Dr R. Dulbecco and Dr G. W. Beadle, and to the Gosney Fund Board of Trustees, the American Cancer Society Inc., and the American Cancer Society, California Division, for financial support as a Research Fellow. I am indebted to the Wright-Fleming Institute of Microbiology, St Mary's Hospital, London, for leave of absence.

REFERENCES

Bauer, D. J. (1952). Metabolic aspects of virus multiplication. In 'The nature of virus multiplication'. *Symp. Soc. gen. Microbiol.* **2**, 46.

Cooper, P. D. (1955). A method for producing plaques in agar suspensions of animal cells. *Virology*, **1**, 397.

Cooper, P. D. (1957*a*). Paths of phosphate transfer in normal chick embryo cells and in cells infected with vesicular stomatitis virus. *J. gen. Microbiol.* **17**, 335.

Cooper, P. D. (1957*b*). An osmotic barrier for inorganic phosphate in chick embryo cells and its stability during the latent and release periods of infection by vesicular stomatitis virus. *J. gen. Microbiol.* **17**, 353.

Dulbecco, R. (1955). Interaction of viruses and animal cells. A study of facts and interpretations. *Physiol. Rev.* **35**, 301.

Dulbecco, R. & Vogt, M. (1954*a*). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **99**, 167.

Dulbecco, R. & Vogt, M. (1954*b*). One-step growth curves of western equine encephalomyelitis virus on chicken embryo cells grown *in vitro* and analysis of virus yields from single cells. *J. exp. Med.* **99**, 183.

Earle, W. R. (1943). Production of malignancy *in vitro*. IV. Mouse fibroblast cultures and changes seen in living cells. *J. nat. Cancer Inst.* **4**, 165.

McClain, M. E. & Hackett, A. J. (1955). Comparison of growth of vesicular stomatitis virus in five tissue culture systems. *Fed. Proc.* **14**, 471.

Melnick, J. C. (1955). Tissue culture techniques and their application to original isolation, growth and assay of poliomyelitis and orphan viruses. *Ann. N.Y. Acad. Sci.* **61**, 754.

Rubin, H., Baluda, M. & Hotchin, J. E. (1955). The maturation of western equine encephalomyelitis virus and its release from chick embryo cells in suspension. *J. exp. Med.* **101**, 205.

Sellers, R. F. (1955). Growth and titration of the viruses of foot-and-mouth disease and vesicular stomatitis in kidney monolayer tissue cultures. *Nature, Lond.* **176**, 547.

Skinner, H. H. & Bradish, C. J. (1954). Exposure to light as a source of error in the estimation of the infectivity of virus suspensions. *J. gen. Microbiol.* **10**, 377.

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COOPER, P. D. (1957). *J. gen. Microbiol.* 17, 335–352

Paths of Phosphate Transfer in Normal Chick Embryo Cells and in Cells Infected with Vesicular Stomatitis Virus

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SUMMARY: The paths of phosphate transfer were compared in normal and vesicular stomatitis virus-infected chick embryo cell monolayers during one-step virus growth. Phosphate entered the normal cell via an inorganic pool in reversible equilibrium with external phosphate, and passed to large-molecule phosphate from this pool or from organic acid-soluble phosphate. During the latent and exponential release periods of virus infection there was no detectable slowing or stimulation of the rate of gain of ^{32}P by acid-soluble inorganic and organic phosphates (AI and AO), lipid phosphate (LP), ribonucleic acid (RNA) and other phosphate fractions until uptake ceased in nearly all fractions about half-way through exponential release. Negligible P or ^{32}P entered or left deoxyribonucleic acid (DNA) in normal or infected cells in this system. Before and during exponential release there was no detectable lysis of nuclei, mitochondria or microsomes (examined after isolation), and no detectable loss of ^{32}P from AI, AO, LP, RNA or DNA, except for a late 30–50 % decrease in the ^{32}P of the sucrose-soluble RNA of disrupted cells. This could be a secondary effect (i.e. onset of cell death) rather than an essential stage of virus growth. Gross lysis was evident in all fractions 20 hr. after infection, with the exception of the acid-soluble inorganic fraction not in reversible equilibrium with the medium.

Viral nucleic acid synthesis should be reflected in some changes in phosphate metabolism. A study of such changes should provide information on virus growth complementary to direct examination of infected cells (e.g. by the electron microscope) which is likely to show stages of maturation rather than of nucleic acid replication. Chemical changes should tell more than relative assays of infective unit content when examining subcellular fractions, as such assays only count subcellular particles with attached mature virus rather than the number or nature of virus attached; non-adsorption of released mature virus to free subcellular particles may not be a good control. Furthermore, this overlooks possible viral replication in the 'soluble' cytoplasmic portion, as infective virus, being particulate, will always be removed from this fraction.

The vesicular stomatitis (VS) virus-chick embryo cell system can satisfy certain criteria for chemical study of the effects of virus growth (Cooper, 1957*a*). Such a study is presented below, where phosphate metabolism is compared in normal and infected cells during the latent and exponential release periods.

There is little data on normal phosphate pathways available for intact animal cells *in vitro* with all cells equally accessible to the medium, and the chick cell system, although definitely limited metabolically, has some technical advantages for such a study over the living animal. In particular, one knows the specific activity of the external phosphate, and one can trace phosphate pathways from the specific activities of increments of homogeneous phosphate fractions (Mitchell & Moyle, 1953). Some information on the entry of inorganic phosphate is presented separately (Cooper, 1957*b*).

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METHODS

Preparation of chick cell monolayers, virus stocks, virus assay, media and the method for the one-step growth curve on monolayers are described by Cooper (1957*a*).

Type of experiments described. In most experiments, one infected and one control monolayer plus media were harvested at intervals to give one-step growth curves lasting less than 24 hr. In the first 2–3 days, non-infected monolayers lost negligible amounts of soluble or particulate RNA or DNA, assimilated ^{32}P maximally, remained normal microscopically and grew virus well so that negligible cell material was lost or changed by 'non-specific' death, lysis or detachment.

Methods of studying P pathways. The words 'exchange', 'gain' or 'loss' are used to define the phosphate transfers, rather than the ambiguous 'turnover'. Three types of experiment were used.

(*a*) '*Specific activities of increments.*' The relative specific activities ('R values'), i.e. the ratios of the observed values of $^{32}P/P$ to that value to be expected at complete equilibration (Mitchell & Moyle, 1953), of some Schmidt-Thannhauser fractions were determined at intervals during uptake of ^{32}P by cells. Then, if a fraction A is homogeneous the specific activity of any small sample passing from A into another fraction B ('specific activity of the increment of B') will have the specific activity of A (Mitchell & Moyle, 1953). Thus, knowing the specific activity of the increment of B, the true precursor pool to B is known if the only alternatives have sufficiently different specific activities. In practice this method is limited, since few pools are homogeneous and a single specific activity of an increment rests on four assays so that errors are magnified. It was only used for normal pathways.

(*b*) '*Gain*' *experiments.* $^{32}PO_4$ was added with the virus (i.e. to monolayers 20 hr. old) and the increase with time of the ^{32}P of each fraction was followed in control and infected cells during one-step virus growth.

(*c*) '*Loss*' *experiments.* $^{32}PO_4$, added to the Petri dishes with the cell suspensions, was incorporated overnight at 37°. After 20 hr. the medium was removed, the cell layers washed three times with warm medium, virus added for one-step growth and the progressive loss of the total ^{32}P of each fraction was followed. The ^{32}P added did not exceed $1\,\mu c$/ml. medium.

Cells were fractionated after 'gain' and 'loss' experiments by the Schmidt-Thannhauser, sucrose and sucrose + $CaCl_2$ cell homogenization, and citric acid methods described below. The two complementary experiments proved quite informative for P pathways in control cells, particularly for differentiating those fractions which were in reciprocal exchange from those which were not.

They should also show virus-induced lysis, or stimulation of incorporation or exchange, which if big enough should affect the total ^{32}P of any fraction in either or both 'gain' or 'loss'.

Harvesting of cells. The medium was frozen at intervals for virus, P or ^{32}P assay, and the cells resuspended from the unwashed monolayer with 5 ml. 25 mg./ml. trypsin (10 min. at 37°) were quantitatively removed, washed once with 5 ml. cold phosphate buffer saline (PBS) and frozen for Schmidt-Thannhauser separation, or washed once with 5 ml. cold 0·25 M-sucrose for homogenization or citric acid extraction.

Sucrose homogenization. The method of Hogeboom, Schneider & Striebich (1953) was used. The washed cells were quantitatively transferred to a Potter-Elvehjem glass homogenizer in sufficient (0·15 ml.) 0·25 M-sucrose to fill the space between pestle and container without overflowing and without air bubbles. The pestle was rotated by an electric motor at an arbitrary constant speed while the container was held in a cradle; the tension was kept constant for all samples by supporting the cradle with stretched rubber bands. Each sample contained 2×10^7 cells, and the cells were always kept at ice-water temperature. The rotation time (usually 60 sec.) allowed at least 95 % cell rupture (haemocytometer count). Recovery of nuclei, counted in 0·1 M-citric acid containing 1 mg./ml. crystal violet, was 75–90 % in sucrose alone, but after addition of 0·0018 M-$CaCl_2$ according to Hogeboom *et al.* (1953) was always more than 95 %. The ruptured suspension was quantitatively removed and centrifuged for 10 min. at 1500 rev./min. to sediment remaining cells and nuclei, either over a 1 ml. layer of 0·34 M-sucrose (giving excellent separation) or directly, and washed once with 2 ml. 0·25 M-sucrose. The pooled supernatant fluids were centrifuged for 10 min. at 10,000 rev./min. in the Spinco rotor No. 40, and the opaque sediment ('mitochondria') washed once with 2 ml. 0·25 M-sucrose. The supernatant fluids, again pooled, were then centrifuged at 25,000 rev./min. for 1 hr., and the almost clear supernatant removed ('soluble fraction'). The translucent pellet ('microsomes') was resuspended in 1 ml. 0·25 M-sucrose without washing. The contamination in the nuclear fraction (calculated from theoretical dilution by washing) was < 3 % of the cytoplasm using sucrose alone, but 30 % of its radioactivity may be due to the 5 % of cells remaining unbroken (better preparations were not possible because of the relative fragility of nuclei). The addition of 0·0018 M-$CaCl_2$ to the sucrose, however, gave very good recoveries of nuclei substantially uncontaminated with whole cells, but in this case mitochondria were not separable from microsomes. Therefore, similar experiments were performed in presence or absence of $CaCl_2$ to examine particularly either nuclei, or mitochondria plus microsomes respectively. The mitochondrial fraction contained < 0·5 % (v/v) of the microsome + soluble fractions, and the microsomes 2–4 % (v/v) of the soluble fraction.

Citric acid fractionation. This method (Sanford, Earle, Evans, Waltz & Shannon, 1951) was as follows. To the pellet of sucrose-washed cells 2 ml. of 0·1 M-citric acid were added and the tubes immersed in a 37° water bath with periodic vigorous pipetting. After 1 hr. the tubes were centrifuged for 10 min. at

1500 rev./min., the supernatant fluid removed and 2 ml. fresh citric acid added. Then after 1 hr. at 37° the tubes were kept at 2° overnight, centrifuged, the supernatant fluids pooled ('citric acid soluble' fraction) and the sediments ('nuclei') resuspended in citric acid for assay. Recovery of nuclei (haemocytometer count with 0·1 % crystal violet) was 80 %, intact cells were <2 %.

Modified Schmidt-Thannhauser separation method. This was developed from Mitchell & Moyle (1953), Davidson, Frazer & Hutchison (1949), Davidson & Smellie (1952) and Katchman & Fetty (1955), and is presented schematically in the diagram. Some abbreviations and definitions, mostly derived from Mitchell & Moyle (1953) are:

'Hot acid hydrolysis' (Katchman & Fetty, 1955) = addition of 1/10 vol. 10 N-HCl, immersed in boiling water for 15 min.

'Mg pptn. = addition to $\frac{1}{2}$ ml. sample of 0·1 ml. 5 % $MgCl_2$ + 0·1 ml. 0·2 M-phosphate (as carrier) + 0·1 ml. conc. ammonia solution (sp.gr. 0·880). After 16 hr. at 2°, the crystals were sedimented in the centrifuge.

'Acid-soluble' = soluble after 30 min. treatment with 10 % (w/v) trichloroacetic acid (TCA) at 2°.

'mI' = inorganic phosphate of the medium.

'mAO' = organic acid-soluble phosphates present in medium but derived from cells.

The remaining fractions are defined as being present in the cell only.

'AI' = acid-soluble inorganic orthophosphate.

'AO' = acid-soluble organic phosphate stable to 'hot acid hydrolysis'.

'7′P' = acid-soluble phosphate converted to AI by 'hot acid hydrolysis' ('seven-minute phosphate', Katchman & Fetty, 1955).

'LMP' = acid-insoluble phosphate (large molecule phosphate).

'LP' = hot-methanol-soluble phosphate (lipid phosphate).

'RNA' = acid-insoluble phosphate transformed to AO by warm alkali, and regarded as predominantly derived from ribonucleic acid phosphate.

'PP' = acid-insoluble phosphate transformed to AI by warm alkali, and including phospho-protein phosphate.

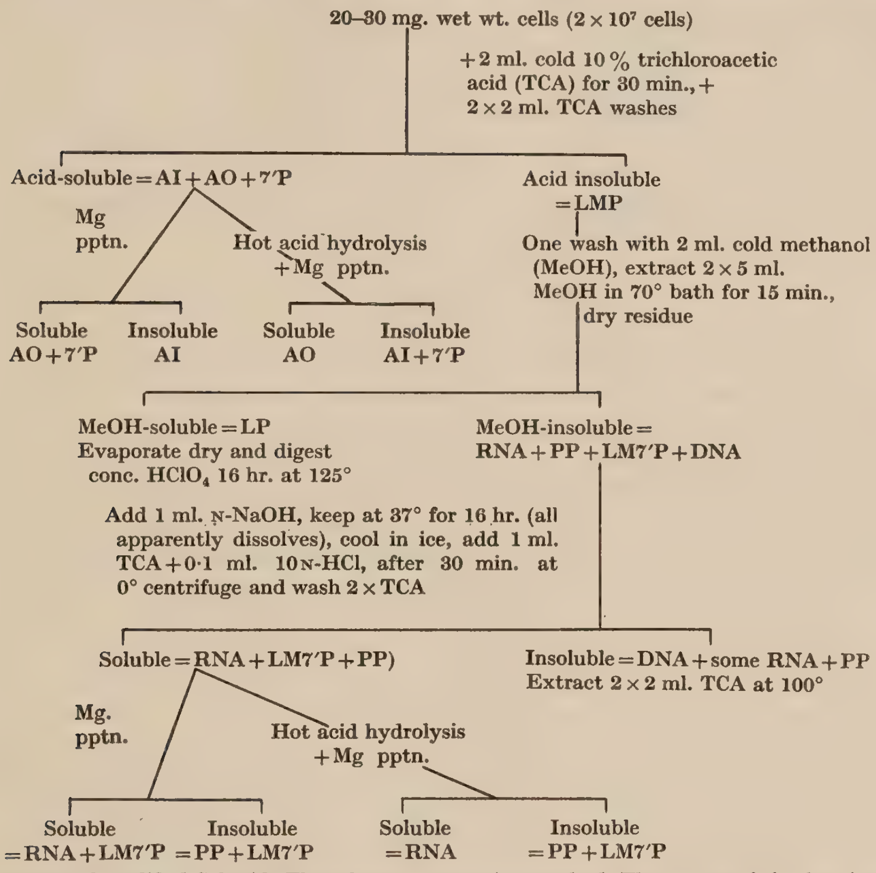
'LM7′P' = acid-insoluble phosphate transformed to '7′P' by warm alkali.

'DNA' = essentially residual phosphate, mostly derived from deoxyribonucleic acid phosphate, but containing some residual RNA and PP phosphates.

In one 'gain' experiment (Fig. 2*c*) RNA was extracted from the cells after harvesting by hot 10 % (w/v) NaCl according to Davidson & Smellie (1952). Extract and residue were subsequently fractionated by the Schmidt-Thannhauser procedure.

Efficiency of the fractionation procedures. It is acknowledged (e.g. Hogeboom *et al.* 1953) that the current methods of fractionating cells into their particulate components are unsatisfactory, and it is clear from the above procedures that, for example, 'mitochondria' will contain a small proportion of microsomes and nuclei, by their aggregation or disintegration, even if each fraction be completely homogeneous with respect to particle size. 'Mitochondrial' and 'microsomal' preparations have been observed to contain at least two

(Laird, Nygaard, Ris & Barton, 1953) or three (Novikoff, Podber, Ryan & Noe, 1953) types of components, respectively, and the 'sucrose-soluble' and citric-acid soluble fractions will clearly contain any extractable substances which properly belong to a particulate fraction. The present work is therefore obliged to define the fractions obtained solely in terms of their mode of preparation. Except for the nuclei, whose purity can be partly observed, much reserve must be attached to interpreting the fractions in terms of their function in the intact cell.


20–30 mg. wet wt. cells (2 × 10⁷ cells)
+2 ml. cold 10 % trichloroacetic acid (TCA) for 30 min., + 2 × 2 ml. TCA washes
Acid-soluble = AI + AO + 7'P
Acid insoluble = LMP
Mg pptn.
Hot acid hydrolysis + Mg pptn.
One wash with 2 ml. cold methanol (MeOH), extract 2 × 5 ml. MeOH in 70° bath for 15 min., dry residue
Soluble AO + 7'P
Insoluble AI
Soluble AO
Insoluble AI + 7'P
MeOH-soluble = LP
Evaporate dry and digest conc. HClO₄ 16 hr. at 125°
MeOH-insoluble = RNA + PP + LM7'P + DNA
Add 1 ml. N-NaOH, keep at 37° for 16 hr. (all apparently dissolves), cool in ice, add 1 ml. TCA + 0·1 ml. 10 N-HCl, after 30 min. at 0° centrifuge and wash 2 × TCA
Soluble = RNA + LM7'P + PP)
Insoluble = DNA + some RNA + PP
Extract 2 × 2 ml. TCA at 100°
Mg. pptn.
Hot acid hydrolysis + Mg pptn.
Soluble = RNA + LM7'P
Insoluble = PP + LM7'P
Soluble = RNA
Insoluble = PP + LM7'P

Scheme of modified Schmidt-Thannhauser separation method. The names of the fractions (e.g., 'RNA') are used by virtue of other workers' results with this method, and serve as definitions rather than as indications that these fractions have been checked chemically by the author.

Estimation of P *and* ^{32}P. Phosphate was determined as inorganic phosphate after 10 hr. digestion at 120° with 0·5 ml. $HClO_4$ by the method of Fiske & SubBarow (1925). ^{32}P was determined after drying the neutralized samples on 1 cm. copper planchettes by means of a Nuclear Instrument and Chemical Corp. (Chicago) automatic end-window Geiger counter with window thickness *c.* 0·5 mg./cm.^{2}. All samples were counted for at least 1000 counts and correction was made where necessary for radioactive decay and background counts

(< 16 cts./min.). Samples were always counted where self-absorption and coincidence were negligible (< 10 mg./cm.^{2} and < 5000 cts./min. respectively).

Preparation of fully-labelled cells. Twenty-five fertile eggs were inoculated via the yolk sac with 20 μc. of ^{32}P in 0·2 ml. of Earle's saline (ES) before incubation. Only 6 embryos survived to 10 days, while the remainder were bacteriologically sterile but only 1 cm. long (fertility rate of un-inoculated eggs was 80 %). At 10 days the full-size embryos were removed, cells prepared and allowed to form as monolayers overnight in the usual way in non-radioactive medium. After 20 hr. the monolayers were washed twice with medium, and the usual one-step growth 'loss' experiment performed, the cells being harvested and fractionated by the Schmidt-Thannhauser procedure. Virus production was, if anything, somewhat higher than average.

RESULTS

Normal pathways of phosphate transfer in monolayers 20–40 *hr. old*

At least five main chemical fractions appeared to exist in each of at least four main subcellular fractions. Few, if any, of the fractions were homogeneous. An attempt is made to trace the broad phosphate pathways among the major chemical (Schmidt-Thannhauser) fractions in the cell as a whole, and from P and ^{32}P contents of centrifugal fractions at particular times certain further details may be deduced. The interpretations summarized in Fig. 1 are given at this stage to help presentation of the evidence.

Source and function of AI. $^{32}PO_4$ passed from the medium into AI much faster than into other fractions, while no net increase of the AIP occurred (Table 1); the transfer had largely ceased after 4–5 hr. (Fig. 2) when the specific activities of AI and mI were substantially equal (Table 1). The implied equal loss of non-radioactive P should be large and rapid and was not found as a gain in any organic fraction, but the reverse loss of AI ^{32}P back to mI occupied the same period of time (Fig. 3) so that evidently at least some of AI is in reversible equilibrium with mI. However, only 30 % (Table 3) to 50 % (Fig. 3) of the AI ^{32}P gained in overnight incubation, and equivalent to about half the AIP of the cells (from Tables 1 and 2*a*) equilibrates in this way, showing that AI is not homogeneous. The two AI fractions can be called 'reversible' (AI_R) and 'irreversible' (AI_I) and appear from these data to have similar specific activities. AI_I can only be distinguished from AI_R as ^{32}P after equilibration with an excess of non-radioactive mIP, when (Fig. 3 and Table 3*a*) the AI_I ^{32}P was twice the highest value of the AO ^{32}P, while the total AI_I P must be considerably less than the total AOP (from Table 1, assuming that AI_I P $\leqslant$ half $[AI_R + AI_I]$). This large difference in specific activity between AO and AI_I only allows that AI_I is rapidly derived from AI_R or mI at a rate similar to the exchange into AI_R, and not from AO or large molecule phosphate (LMP). Its stability in the 'loss' experiments, even after 20 hr., suggests that it is not an intermediary in P metabolism. It may be a very acid-labile polyphosphate; its stability to the presumably enzymic lysis in infected cells up to 20 hr. (Fig. 3) does not suggest an organic phosphate.

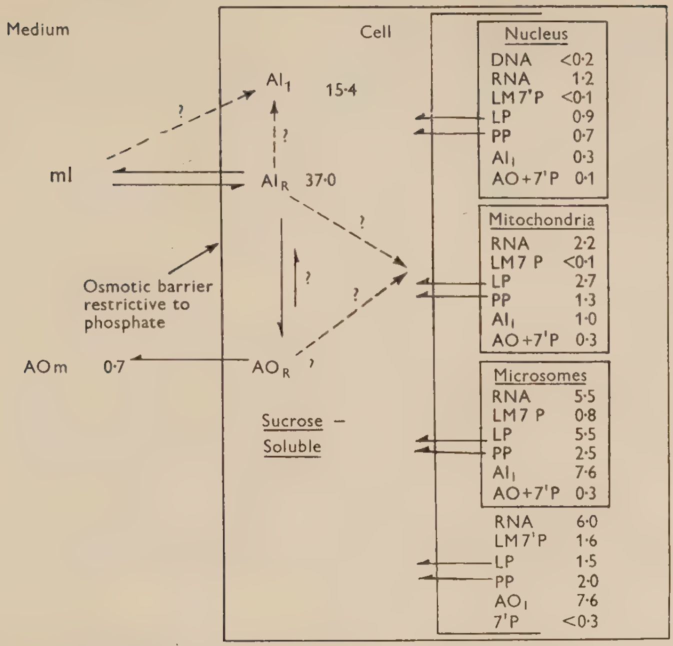

Medium
Cell
Nucleus
DNA <0·2
RNA 1·2
LM7'P <0·1
LP 0·9
PP 0·7
AI₁ 0·3
AO+7'P 0·1
AI₁ 15·4
?
AI_R 37·0
ml
Mitochondria
RNA 2·2
LM7 P <0·1
LP 2·7
PP 1·3
AI₁ 1·0
AO+7'P 0·3
Osmotic barrier restrictive to phosphate
AOm 0·7
AO_R
Microsomes
RNA 5·5
LM7 P 0·8
LP 5·5
PP 2·5
AI₁ 7·6
AO+7'P 0·3
Sucrose – Soluble
RNA 6·0
LM7'P 1·6
LP 1·5
PP 2·0
AO₁ 7·6
7'P <0·3

Fig. 1. Diagram of suggested phosphate pathways. Evidence for the restrictive osmotic barrier is given elsewhere (Cooper, 1957*b*). Figures represent distribution of ^{32}P after a 'loss' experiment of 7 hr. (data from Table 3*a*) calculated as percentage of total ^{32}P in washed cells at commencement of 'loss'.

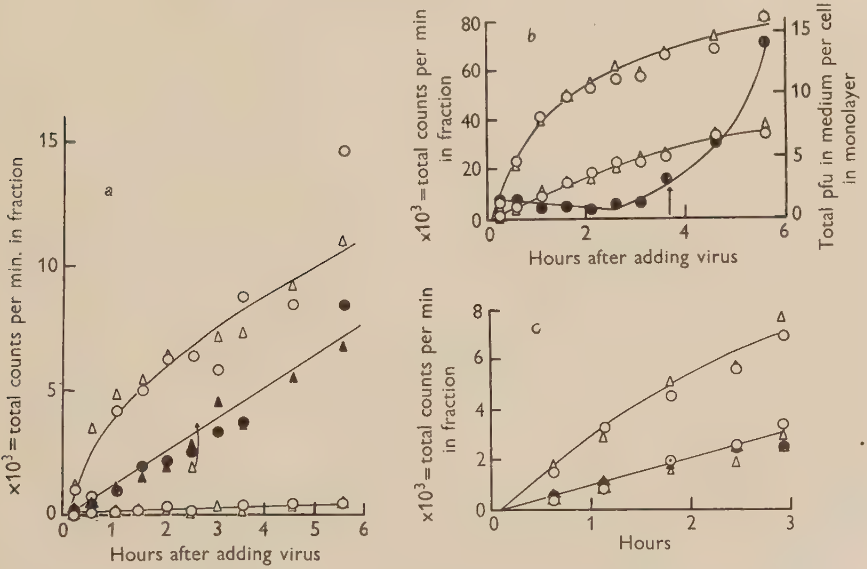


Fig. 2. A ^{32}P 'gain' experiment (*a* and *b*) followed by Schmidt-Thannhauser (S-T) fractionation of whole cells. Circles represent control, and triangles infected samples. ^{32}P was added 5 min. after addition of virus. In (*a*), the topmost and lowest curves (○, △) represent RNA and DNA respectively, and the middle curve (●, ▲) LP. In (*b*), the upper and lower curves (○, △) represent AI and AO respectively, and (●) = virus release. Multiplicity of adsorption = 2–3. The arrow (*b*) indicates the end of the latent period obtained by extrapolation of a logarithmic plot. (*c*) Represents a separate experiment performed identically with (*a*) and (*b*). The upper curve in (*c*) is total S-T RNA; the lower is the result of extraction with hot 10 % NaCl followed by separation of S-T RNA from the extract (○, △) and the residue (●, ▲).

Table 1. *'Relative specific activities' (R), total phosphorus (µg. P) and total ^{32}P (counts per min = c/m) contents of Schmidt-Thannhauser phosphate fraction during ^{32}P incorporation by chick-cell monolayers maintained in ES/4 % horse serum at 37° in a CO_2-air mixture*

$^{32}PO_4$ was first added to the once-washed monolayers 20 hr. after their formation, and each sample represents one monolayer (2 × 10⁷ cells).

Hours after addition of $^{32}PO_4$	mI			$AI_{(R+I)}$			$AO_{(R+I)}$			LP		
	c/m × 10³	µg. P	R	c/m	µg. P	R	c/m	µg. P	R	c/m	µg. P	R
0·1	234	190	1·29	342	7·4	0·049	145	11	0·014	0	6·6	0
2·66	—	—	—	3580	7·4	0·50	935	17	0·058	137	7·7	0·018
5·17	219	175	1·32	6960	7·4	0·99	2520	19	0·14	433	8·6	0·053
7·58	206	184	1·18	8000	9·0	0·94	3060	15	0·21	640	7·4	0·091
11·42	—	—	—	6760	5·4	1·32	3760	19	0·21	1125	6·6	0·18
22·66	182	188	1·07	7970	5·0	1·63	5330	20	0·28	2080	6·3	0·35
27·5	202	224	0·95	8220	5·2	1·66	6780	27	0·26	3380	6·5	0·38

Hours after addition of $^{32}PO_4$	RNA + PP + LM7'P			DNA			Total cell		
	c/m	µg. P	R	c/m	µg. P	R	c/m	µg. P	R
0·1	52	11·4	0·005	0	3·2	0	539	39·6	0·014
2·66	381	12·2	0·033	14	2·3	0·006	5,047	41·2	0·13
5·17	960	13·0	0·078	43	2·1	0·024	10,916	50·1	0·23
7·58	1113	13·3	0·088	45	2·0	0·024	12,858	46·7	0·29
11·42	1570	11·0	0·15	75	2·0	0·045	13,290	44·0	0·32
22·66	2820	12·8	0·23	91	2·0	0·054	18,291	46·2	0·42
27·5	3650	14·2	0·27	98	2·2	0·053	22,128	55·3	0·42

In the 'loss' experiment of Fig. 3 it can be seen that the gain in mI ^{32}P is slightly greater than the drop of AI_R, which is unexpected as some AI_R is lost to AO and LMP by synthesis; it is probably due to the small loss from LP, PP and possibly AO noticeable in Table 2 and Fig. 3.

As with *Micrococcus pyogenes* (*Staphylococcus aureus*) (Mitchell & Moyle, 1953) AI_R here is probably a pool supplying most of the cellular P; the R values of the increase of AO and LMP mentioned in the next sections suggests that AI is an intermediary between mI and organic phosphate. The way in which this pool is retained by the cell is discussed separately (Cooper, 1957*b*).

Source and function of AO. The increment in AO between the mean values for 0–8 hr. in Table 1 (1662 cts./min. and 15·5 µg. P) and 11–28 hr. (5290 cts./min. and 22 µg. P) is 3628 cts./min. and 6·5 µg. P, so that its R value is 0·59; this amount of phosphorus at such a specific activity is clearly only derivable largely from mI or AI, whose specific activity is about unity between these times. Some P may be derived from the relatively unlabelled LMP by lysis or acid-lability. This same figure of 0·59, rather than a value nearer one, and the low specific activities of the initial increments make it likely that most AO derives from AI rather than mI.

In some 'gain' experiments the R values of the increments of AO were much higher than 1 (2–3), indicating a high rate of replacement of P by ^{32}P;

while LMP was increasing and was low in ^{32}P this could be due to AO acting as intermediary between AI and LMP, or by reciprocal exchange between AI and AO. Such an exchange is not suggested by the loss experiment of Fig. 3, where the drop in AO ^{32}P is not reciprocated by a gain in LM ^{32}P but by a gain in mAO ^{32}P. On the other hand, much less AO ^{32}P was lost to the medium in the experiment of Table 3*a*. The stability of about half the AO ^{32}P even after 23 hr. in this experiment (AO_I) suggests that not all the AO is available as a metabolic pool.

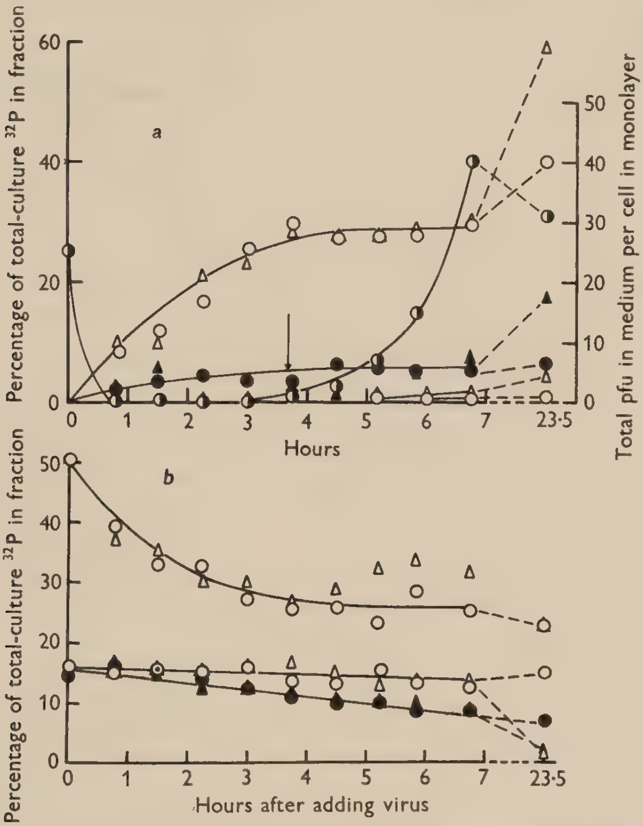


Fig. 3. A ^{32}P 'loss' experiment followed by Schmidt-Thannhauser fractionation of whole cells. Circles represent control and triangles infected samples. (*a*) Shows changes in the medium and (*b*) changes in the cells. In (*a*), the upper (○, △) = AI, the lower (○, △) = LMP, (●, ▲) = AO and (◑) = virus release. In (*b*), the upper (○, △) = AI, the lower (○, △) = RNA, and (●, ▲) = AO. Multiplicity of adsorption = 2–3. The arrow indicates the end of the latent period obtained by extrapolation of a logarithmic plot. Cellular lipid phosphate and phospho-protein behaved similarly to RNA + LM7′P. 'DNA' is not given, as this was uniformly 0·3–1·2 % in both control and infected cells at all times.

It is possible that at least some AO is a part of LMP easily separated by TCA rather than, like inorganic phosphate (Cooper, 1957*b*) free as small molecules in the cell; in this case the particular acid-labile LMP is restricted to the sucrose-soluble fraction (Table 3*a*).

It is interesting that the bulk of the P accumulates in the AO (Table 1), and is therefore reminiscent of *Micrococcus pyogenes* in absence of nitrogen source (Mitchell & Moyle, 1953); it is as though the major limitation in the P syntheses of this system occurs at the stage of incorporation into LMP, particularly DNA (see below). However, this appears to be no deterrent to virus growth.

Source of LP and RNA + PP + 7′P. It is difficult to indicate the sources of these fractions because of the presence of components which are able to equilibrate with AI, particularly in the LP and PP fractions (Table 2), and which therefore produce the high R values of increments characteristic of high exchange rates. This is particularly so for the specific activity of the mean increment between the first half (0–7 hr.) and the second half of the experiment of Table 1, and was even more noticeable if either serum or both serum and glucose were omitted, when marked lysis of LMP occurred. On the other hand, the R values of the early increments (when the cells were in better condition) of LP and RNA + PP + 7′P were all low, suggesting that the more stable portions of these fractions (particularly RNA) are derived from AO rather than AI or mI. The evidence, however, is not good for this; it is unsatisfactory to base any estimation of the source of RNA P on the specific activities of heterogeneous groups of substances, such as 'AO' and 'RNA', rather than the individual nucleotides presumed to be concerned. It is clear from the low activity of DNA that RNA P cannot mainly be derived from DNA P. It is noteworthy that the exchange rate of LP is much lower than that of *Micrococcus pyogenes* (Mitchell & Moyle, 1953), where LP may be concerned with phosphate uptake mechanisms.

Source of DNA. This cannot be determined from these experiments, since very little DNA was synthesized under the conditions used. It is quite likely that the bulk of the ^{32}P observed in the 'DNA' fraction is due to the contaminating residue of RNA left by the somewhat inefficient alkaline hydrolysis (except in Table 2), and the scatter frequently present in 'DNA' ^{32}P supports this. It should be noted that the lack of DNA incorporation is probably due to the large number of cells added to each plate (2×10^7), as less than this number can be seen to increase in numbers.

Comparison of P metabolism of normal and virus-infected cells

As the R values of increments are too inaccurate to detect small changes, the complementary 'gain' and 'loss' experiments are compared during one-step growth. Stimulation, blockage or reversal of the numerous P pathways by virus infection might be found by various fractionations.

Schmidt-Thannhauser fractionation of whole cells. The 'gain' experiments (Fig. 2) showed that VS infection had no detectable effect during the latent and early release phases on ^{32}P uptake in AI, AO + 7′P, LP, RNA + PP or 'DNA' fractions; no effect in the bulk RNA fraction was found when it was extracted with 10% NaCl (Fig. 2*c*) by the method of Davidson & Smellie (1952). AI equilibration with mI was unaffected during 'loss' experiments (Fig. 3, Table 2) (see also Cooper, 1957*b*) and so was total DNA. A barely significant increase in AI about the end of the latent period (Fig. 3) may be derived from the LMP and particularly the RNA + LM 7′P fraction (Table 2*a*). After 23 hr. the ^{32}P of the infected cells was nearly all in the medium, mostly as inorganic and acid-soluble organic phosphates. There was a very significant increase in medium LMP (Fig. 3) which coincided with virus release; the $\mathrm{AI_I}$ was not lysed. The use of fully labelled cells (Table 2) takes into account fractions (such as the DNA) which are not fully labelled by incorporation into monolayers as in Fig. 3.

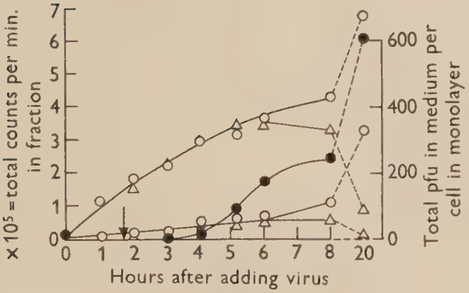


Fig. 4

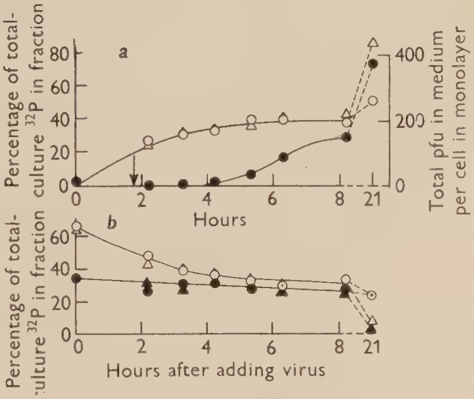


Fig. 5

Fig. 4. A ^{32}P 'gain' experiment followed by cell fractionation by the citric acid method. (●) = virus release. Open circles represent control and triangles infected samples, the upper curve of which shows the citric acid-soluble fraction and the lower the nuclei. The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5–6.

Fig. 5. A ^{32}P 'loss' experiment followed by cell fractionation by the citric acid method. Circles represent control and triangles infected samples. (*a*) shows virus release (●) and release of ^{32}P to the medium. (*b*) shows the citric-acid soluble fraction (○, △) and the nuclei (●, ▲). The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5–6.

0·1 M-*Citric acid fractionation.* This method in my hands separated an impure nuclear fraction (cell-free but with attached wisps of cytoplasm) and a citric-acid soluble fraction, comprising the acid-soluble nuclear components (small, Table 3) plus the rest of the cell. 'Gain' experiments employing this fractionation (Fig. 4) showed that total P uptake ceased in both fractions during the exponential release period, while the complementary 'loss' experiment (Fig. 5) showed no big loss from nuclei or cytoplasm as a whole. Increased ^{32}P release into the medium during this period was equivocal but was marked after 24 hr., both nuclei and soluble fractions being much depleted in both experiments. Unstained nuclei isolated towards the end of exponential virus release were microscopically identical with controls, the nuclear membrane showing no gross damage.

Potter-Elvehjem homogenization. Cells were either homogenized in 0·25 M-sucrose, giving good separation of mitochondria and microsomes but allowing easier rupture of nuclei (Hogeboom *et al.* 1953); or in 0·25 M-sucrose + 0·0018

Table 2. *Effect of vesicular stomatitis virus infection on the rate of ^{32}P loss (one step 'loss' experiment) from monolayers made from embryos uniformly labelled* in ovo

Multiplicity of adsorption = 4·5 pfu/cell. Table 2 (*a*) changes in cellular ^{32}P, (*b*) changes in medium ^{32}P. The monolayers had been formed for 20 hr. in non-radioactive medium and washed, so that AI_R ^{32}P and AO_R ^{32}P had been removed before addition of virus. C = control monolayers, V = virus-treated monolayers. Figures represent a percentage of the total ^{32}P of a culture (medium + cells).

Table 2*a*

Hours after inoculation	AI_I		AO_I		LP		PP		RNA + LM7′P		DNA		pfu/cell present in medium
	C	V	C	V	C	V	C	V	C	V	C	V	
0	5·6	5·6	7·2	7·2	24·2	24·2	24·4	24·4	16·1	16·1	22·5	22·5	7·5
1·3	4·0	4·0	7·7	9·0	23·7	22·5	19·2	21·9	15·9	16·1	22·1	23·3	3·0
2·6	5·1	5·5	7·1	6·4	20·9	20·7	19·9	17·3	16·7	15·6	19·1	21·5	3·2
3·8	2·6	4·2	8·4	7·5	18·6	21·1	15·4	15·8	16·4	16·9	20·9	19·2	14·2
5·0	4·9	4·5	6·1	6·6	20·3	16·0	17·7	26·2	15·6	11·8	18·8	17·7	54·5
6·3	2·5	2·1	8·1	8·4	19·3	18·6	17·7	21·0	14·4	11·8	20·2	19·5	132
7·5	5·1	5·0	5·8	7·3	16·8	17·2	12·7	17·9	15·9	13·6	20·2	18·9	347

Table 2*b*

Hours after inoculation	AIm		AO_m + 7′P		LP		PP + RNA + LM 7′P		DNA	
	C	V	C	V	C	V	C	V	C	V
0	0	0	0	0	0	0	0	0	0	0
1·3	0	0	6	0	3	2	3	0	1	1
2·6	0	4	9	0	3	4	5	4	0	1
3·8	4	2	6	4	5	4	3	5	0	1
5·0	2	7	8	3	2	2	2	2	2	1
6·3	9	8	2	2	2	2	3	2	1	2
7·5	14	11	4	4	3	3	2	3	1	1

M-$CaCl_2$, in which nuclei are less fragile but mitochondria and microsomes are not separable. The gain experiment in Ca-free sucrose (Fig. 6) showed that ^{32}P uptake by microsomes and soluble fraction was halted at the beginning of or during exponential virus release, although mitochondria were not affected. In this experiment the whole cells present in the nuclear fraction masked any changes there. The corresponding 'loss' experiment (Fig. 7) showed no change in nuclei, mitochondria or microsomes, but there was a small drop in the sucrose-soluble fraction and a small increase in the medium during virus release, both barely significant. The 2–3 % more of total ^{32}P regularly found in the medium of infected cells (mostly as inorganic P) would be apparent as a loss in the curves for the particulate fractions if it derived from them, but it would be masked if it were from the soluble fraction.

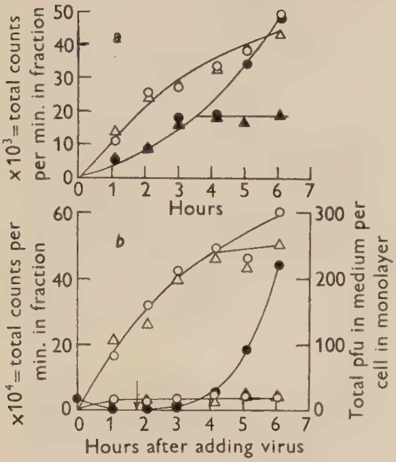


Fig. 6

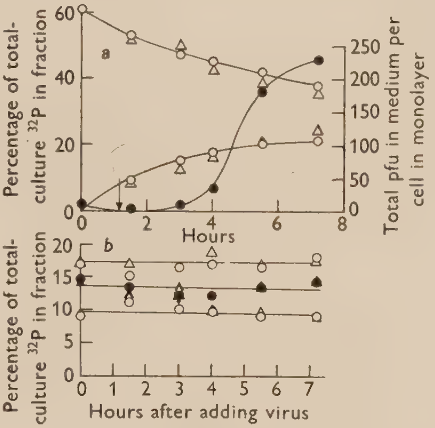


Fig. 7

Fig. 6. A ^{32}P 'gain' experiment followed by cell fractionation by homogenization in Ca-free 0·25 M-sucrose. Circles represent control and triangles infected samples. (*a*) shows 'mitochondria' (○, △) and 'microsomes' (●, ▲); (*b*) shows the sucrose-soluble fraction (○, △, upper curve), nuclei (○, △, lower curve) and virus release (●). The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5–6.

Fig. 7. A ^{32}P 'loss' experiment followed by cell fractionation by homogenization in Ca-free 0·25 M-sucrose. (*a*) shows the sucrose-soluble fraction (○, △, upper curve), ^{32}P release into the medium (○, △, lower curve), and virus release (●). (*b*) shows microsomes (○, △, upper curve), mitochondria (○, △, lower curve) and nuclei (●, ▲). Circles represent control and triangles infected samples. The arrow indicates the end of the latent period obtained from extrapolation of a logarithmic plot. Multiplicity of adsorption = 5–6.

The sucrose-$CaCl_2$ 'loss' experiment is not presented, as no changes occurred in any control or virus fraction (particularly nuclei) up to a release of 135 pfu/cell.

With or without $CaCl_2$, the isolated nuclei were microscopically unchanged by virus release, and the microsomal and mitochondrial pellets after centrifugation were identical with controls.

Table 3*a*. *Effect of VS infection on the Schmidt-Thannhauser (S-T)* ^{32}*P distribution in the fractions obtained by Potter-Elvehjem cell-homogenization in* 0·25 M-*sucrose*

Cells were harvested from 6 control and 6 infected monolayers 7 hr. after the initiation of a one-step 'loss' experiment (multiplicity of adsorption = 6 pfu/cell, titre at harvesting = 3·88 × 10^8 pfu/ml. or 97 pfu/cell, a typical yield for 3-times washed monolayers). Figures represent per cent total ^{32}P of culture (medium + cells) present in that fraction for C = control and V = virus-treated monolayers. $P=\frac{(V-C)}{C}\times 100$ with C and V calculated to 3 decimals.

S-T Fraction	Nuclei			Mitochondria			Microsomes		
	C	V	P	C	V	P	C	V	P
AI	0·34	0·36	+6·76	1·01	1·11	+10·5	0·99	0·81	−18·2
AO + 7′P	0·15	0·15	+2·07	0·31	0·39	+27·6	0·30	0·24	−19·7
LP	0·91	0·85	−5·73	2·66	2·18	−18·2	5·48	4·82	−10·2
RNA	1·24	1·21	−2·42	2·24	2·18	−2·68	5·54	4·73	−14·6
PP	0·66	0·68	+3·35	1·33	1·31	−1·40	2·50	2·02	−19·2
LM 7′P + PP	0·47	0·72	+53·3	1·36	1·57	+15·5	3·36	3·71	+10·1
DNA	0·24	0·47	+9·85	0·16	0·39	+140	0·27	0·23	−16·1
Total	3·33	3·78	+13·5	7·79	7·76	−0·38	15·9	14·5	−8·67

S-T Fraction	Sucrose-soluble			Medium			Total		
	C	V	P	C	V	P	C	V	P
AI	15·4	16·8	+8·89	37·0	38·9	+5·13	54·8	58·0	+5·88
AO + 7′P	7·20*	7·23*	+0·43*	0·74	0·91	+23·0	8·69	8·92	+2·65
LP	1·53	1·65	+8·45	0·30	0·24	−20·1	10·9	9·75	−10·38
RNA	6·00	4·22	−29·7	0·08	0·24	+193	15·1	12·6	−16·8
PP	2·05	1·71	−16·3	0·018	0·088	+396	6·55	5·81	−11·32
LM 7′P + PP	3·67	2·90	−21·0	—	—	—	—	—	—
DNA	1·06	0·66	−37·8	0·005	0·007	+40·6	1·73	1·76	+1·76
Total	34·8	33·8	−3·83	38·2	40·4	+5·84	931†	928†	−0·34†

* Not significantly altered after removal of 7′P. † × 10^3 = total counts per min. in culture.

Table 3*b*. *Some relationships between pfu and LM* ^{32}*P in the medium of the experiment described in Table* 3*a*, *after* 10 *min. centrifugation at* 1000 *rev./min.*

The LM^{32}P was separated by precipitation and washings with TCA.

Treatment	Total pfu in supernatant	Total counts/min. in LM ^{32}P fraction of supernatant	Recovery of pfu (%)	Recovery of LM ^{32}P (%)
(1) After 10 min. at 1000 rev./min.	10·2 × 10^8	504	100	100
(2) 100 mg. washed infusorial earth added to 5 ml., stirred for 5 min. and centrifuged for 2 min. at 1000 rev./min. Supernatant removed for assay	6·41 × 10^8	273	63·2	54·2
(3) 100 mg. fresh infusorial earth added to supernatant, centrifuged 16,000 rev./min. for 30 min. Supernatant removed for assay	0·323 × 10^8	24·4	3·7	5·6
(4) Sediment from (3) resuspended in 5 ml. ES/Ox, centrifuged 2 min. at 1000 rev./min. Supernatant removed for assay	1·47 × 10^8	363	17·2	77·7

The sucrose-soluble RNA during virus release. The unchanged total particulate ^{32}P (gain or loss) and the just significant changes in sucrose-soluble and Schmidt-Thannhauser ^{32}P (loss) made more information necessary, particularly regarding RNA. For this purpose the cells from six control and six infected monolayers of a 'loss' experiment were removed and pooled 7 hr. after infection, fractionated (Potter-Elvehjem) in 0·25 M-sucrose, and the centrifuged products further treated according to Schmidt-Thannhauser. Table 3*a* shows the ^{32}P content of each fraction. The handling errors are less than might be expected, as the recoveries of total ^{32}P differed between control and infected cells only by 0·34 %. The standard deviations of the ratio of difference between infected and control to control of all the experimental values (excluding 'DNA', which is probably RNA contamination and liable to scatter, and medium LMP, in which the infected is much higher than control) was 17·3 and the mean was −4·3. This value of the mean close to zero may indicate that, except for 'DNA', no big errors exist. Of the infected RNA fractions, which should show largest virus effects, the biggest change was the drop in sucrose-soluble RNA ($p = 0·05$). One cannot be sure of such small differences, however. The bulk of the LMP in the medium after low-speed centrifugation sedimented at the same rate as the pfu (Table 3*b*).

Table 4. *Distribution of* ^{32}P *between sucrose-soluble and insoluble fractions after freezing and thawing intact cells and also particulate fractions of cells previously separated by the Potter-Elvehjem method* (0·25 M-*sucrose*)

Cells were harvested and washed after 20 hr. incorporation of ^{32}P. Each fraction was rapidly frozen in dry ice and allowed to thaw at 30°, then centrifuged for 10 min. at 1000 rev./min. ('slow spin') and the sediment (sucrose-insoluble) washed once in 1 ml. sucrose and the supernatants (sucrose-soluble) pooled. Portions of the freeze-thawed cell and mitochondria + microsome fractions were also centrifuged for 1 hr. at 25,000 rev./min. ('fast spin'). 'TCA' = two treatments with cold 10 % trichloroacetic acid.

	Percentage cellular ^{32}P after freeze-thaw in sucrose			
	Sucrose-soluble		Sucrose-insoluble	
Nature of the fraction to be frozen	TCA-soluble	TCA-insoluble	TCA-soluble	TCA-insoluble
Whole cells (slow spin)	66·2	15·5	5·5	12·8
Whole cells (fast spin)	59·9		40·1	
Nuclei (slow spin)	1·05	0·17	4·18	10·2
Mitochondria + microsomes Slow spin	6·27	11·24	2·22	6·83
Fast spin	2·62	1·68	1·84	19·2
Sucrose soluble (slow spin)	52·0	6·46	<0·1	<0·1

In order to confirm this small drop the sucrose-soluble RNA was followed during gain and loss experiments. Cell rupture by one freeze-thaw in sucrose gave a similar soluble fraction (Table 4) to that from Potter-Elvehjem rupture; some extra material may dissolve from subcellular particles, but freeze-thawing the Potter-Elvehjem soluble fraction made nothing insoluble. The sucrose-soluble fraction was therefore prepared by freezing and thawing the intact cells in 0·25 M-sucrose for these experiments. Fig. 8 shows a decrease of 30–50 % in the sucrose-soluble RNA in loss and gain experiments, which is probably significant, with the complete halt of ^{32}P uptake during gain also found in most other 'gain' fractions. No early stimulation of uptake occurred.

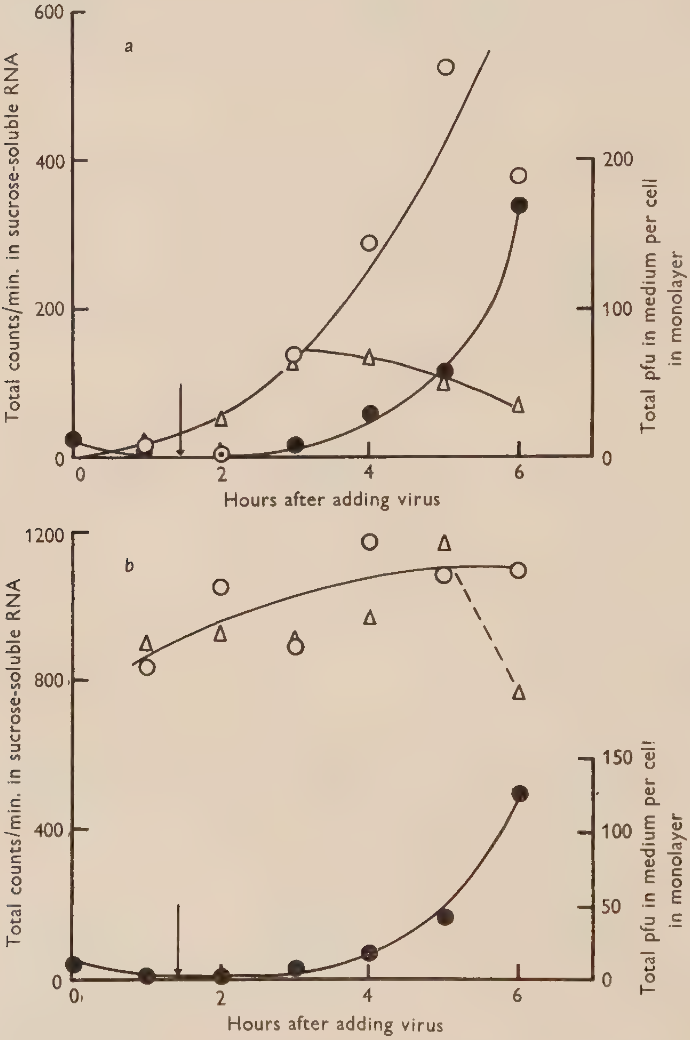


Fig. 8. A ^{32}P 'gain' (*a*) and a ^{32}P 'loss' (*b*) experiment performed on the same batch of plates, followed by preparation of the sucrose-soluble fraction by freezing and thawing in 0·25 M-sucrose and centrifuging for 1 hr. at 25,000 r.p.m. The supernatant was separated by the Schmidt-Thannhauser procedure to obtain alkali-labile acid-insoluble phosphate which was then freed from phospho-protein phosphate and LM 7′P. The remainder was assayed as sucrose-soluble RNA ^{32}P (○, △), where circles represent control and triangles infected samples. (●) = virus release. The arrows indicate the ends of the latent periods obtained from extrapolations of logarithmic plots. Multiplicity of adsorption = 5–6.

DISCUSSION

The normal phosphate pathways, indicated in outline above, are complex, as may be expected. In general, phosphate entered the cell as inorganic phosphate via the inorganic pool which was in equilibrium with the external medium, and seemed likely to enter RNA in particular via AO_R rather than direct from AI_R. However, both organic fractions were heterogeneous, and it is not correct to deduce the RNA source without examining individual nucleotides.

The present data do not indicate which nucleic acid fraction was concerned with virus growth. The lack of DNA synthesis in control and infected cells, while virus increased at least as well as in growing cells, may be a useful simplification in that some pathways were but little used; otherwise it indicates that any viral DNA synthesis (equivalent to less than 0·5 % of the DNA of the cells), or conversion of cellular DNA to viral material which was subsequently released, was too small to be detected. The several RNA's appear similar in that no marked stimulation of incorporation was noticeable either in bulk RNA, sucrose-soluble RNA or in the total ^{32}P of the centrifugal fractions, and no loss occurred in any particulate RNA fraction after virus release. The intact appearance of the isolated cell nuclei after virus release indicates that, if growth occurred intranuclearly, then release from the nucleus cannot occur as a 'burst'. The same applies from the chemical data to the intact cell and to all cell particulates.

Nevertheless, certain changes did occur, the significance of which is not immediately apparent. There was a general halt in P uptake into all fractions (save possibly the mitochondria) soon after 1 pfu was released per infected cell. The small somewhat equivocal decrease in sucrose-soluble RNA P was nevertheless apparent in all experiments designed to show it, and probably represents a lysis as there was a corresponding but barely significant increase in small molecular phosphate. Its appearance as RNA in any other fraction, including the medium, would have been apparent. The significance of this for virus growth may be slight, however, as it is pointed out (Cooper, 1957*a*) that, due to the effect of multiplicity of infection on latent period, a small proportion of the cell population will be at a considerably more advanced stage of viral development than the average. This small lysis may therefore represent the first stage of degeneration in this minority. A probably more significant finding is the presence of RNA ^{32}P in the medium which is adsorbed to infusorial earth and centrifuges at the same rate as the pfu. Some rearrangements of the cellular ribonucleotide ratios have been found during VS infection of chick cells (Dr G. L. Turco, personal communication).

Clearly more data is required to interpret these findings. The fact that the total P content of the matured infective virus must be small compared with the whole cell need not necessarily lead us to expect small changes in the cell. It is necessary to know among other things whether the latent period involves quiescent organization or vigorous viral growth, whether the virus particle contains DNA or RNA and how much, what is the ratio of active to inactive particles in the final yield, and which fraction is entered by invading virus nucleic acid. Such information would then guide the more detailed chemical work required to disentangle the small changes incurred by virus action in this system.

It is a question for further work as to whether a cell-virus system showing bigger changes is necessarily a better model for examination, since such changes may be non-specific, and may serve only to obscure changes relevant to virus synthesis.

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REFERENCES

Cooper, P. D. (1957*a*). Some characteristics of vesicular stomatitis virus growth-curves in tissue culture. *J. gen. Microbiol.* **17**, 327.

Cooper, P. D. (1957*b*). An osmotic barrier for inorganic phosphate in chick embryo cells and its stability during the latent and release periods of infection by vesicular stomatitis virus. *J. gen. Microbiol.* **17**, 353.

Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1949). Phosphorus compounds in the cell. 1. Protein-bound phosphorus fractions studied with the aid of radioactive phosphorus. *Biochem. J.* **49**, 311.

Davidson, J. N. & Smellie, R. M. S. (1952). Phosphorus compounds in the cell. 2. The separation by ionophoresis on paper of the constituent nucleotides of ribonucleic acid. *Biochem. J.* **52**, 594.

Fiske, C. H. & SubBarow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1953). Localisation and integration of cellular function. *Cancer Res.* **13**, 617.

Katchman, B. J. & Fetty, W. O. (1955). Phosphorus metabolism in growing cultures of *S. cerevisiae*. *J. Bact.* **69**, 607.

Laird, A. K., Nygaard, O., Ris, H. & Barton, A. D. (1953). Separation of mitochondria into two morphologically and biochemically distinct types. *Exp. Cell Res.* **5**, 147.

Mitchell, P. & Moyle, J. (1953). Paths of phosphate transfer in *M. pyogenes*: phosphate turnover in nucleic acids and other fractions. *J. gen. Microbiol.* **9**, 257.

Novikoff, A. B., Podber, E., Ryan, J. & Noe, E. (1953). Biochemical heterogeneity of cytoplasmic particles isolated from rat liver homogenates. *J. Histochem. Cytochem.* **1**, 27.

Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K. & Shannon, J. E. (1951). The measurement of proliferation in tissue culture by enumeration of cell nuclei. *J. nat. Cancer Inst.* **11**, 773.

(*Received* 28 *February* 1957)

COOPER, P. D. (1957). *J. gen. Microbiol.* **17**, 353–362

An Osmotic Barrier for Inorganic Phosphate in Chick Embryo Cells and its Stability during the Latent and Release Periods of Infection by Vesicular Stomatitis Virus

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SUMMARY: An osmotic barrier for phosphate very near the visible surface of the chick-embryo cell appears to regulate the reciprocal exchange of inorganic orthophosphate between the medium and a component of the acid-soluble inorganic phosphate of cells kept in monolayer culture. Some non-reciprocal transfer of inorganic phosphate occurs, which may or may not be due to cell damage, and the apparent phosphate-impermeable volume decreases by half after 3 hr. contact with phosphate at 0°. At 2° the exchange is at least 97 % inhibited. The phosphate-impermeable volume after cell rupture is less than 10 % of the intact-cell value, when internal phosphate is released in a form shown by mild separation to be mostly inorganic orthophosphate. Cells lose internal phosphate more slowly in absence of external phosphate, and the addition of external phosphate increases the loss rate to a maximum at physiological concentrations and higher.

Such a barrier provides information on the nature of the cell surface, and shows that adsorption, penetration and release of vesicular stomatitis virus occurs without any detectable damage to the surface controlling the phosphate-exchange rate.

Workers generally agree (e.g. Kamen & Spiegelman, 1948; Sacks, 1948; Mitchell & Moyle, 1953) that concentration of inorganic phosphate into cells does not depend much on simple diffusion alone, but also involves enzymic mechanisms which are possibly phosphorylative. The major uncertainties reside in whether (*a*) these are exclusively surface mechanisms, so that internal orthophosphate is free within an acid-labile osmotic barrier, or whether (*b*) the phosphate diffuses freely, to be immobilized enzymically by some intracellular acid-labile matrix. Sacks (1948) believed the evidence indicated possibility (*a*) for animal cells, but he considered only the alternative of simple physical diffusion (i.e. with no enzymic mechanism) rather than (*b*). Similar possibilities have been discussed by Mitchell (1953) for *Micrococcus pyogenes* (*Staphylococcus aureus*) under the titles of (*a*) exchange-diffusion and, (*b*) exchange adsorption, although simple physical adsorption does not apply in the cases discussed below. Exchange diffusion was preferred, as the cells appeared completely impermeable to phosphate in the cold, but freely permeable when the osmotic barrier was destroyed by acid or 5 % (v/v) butanol in water. Some substances inhibited exchange at concentrations too low for stoichiometric displacement of adsorbed phosphate, but this does not disprove intracellular fixation, as it only indicates that a catalytic rather than a physical adsorption fixation mechanism is operating. More recent evidence is available to indicate that phosphate and other ions exist in the free state within an osmotic barrier in bacteria (Mitchell & Moyle, 1956).

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Greenberg (1952) felt at that time that evidence for the mechanism of phosphate uptake by animal cells was inconclusive. The data presented below seem more definitely in favour of the existence of an osmotic barrier for orthophosphate close to the chick-embryo cell wall, through which by definition phosphate ions do not pass freely but require a transport mechanism that turns out to be very dependent on temperature. Such a barrier can provide information on superficial aspects of the viral growth cycle such as adsorption, penetration and release of the virus. Evidence is presented here that these processes during infection by vesicular stomatitis (VS) virus do not detectably affect the rate of phosphate exchange, suggesting that the phosphate barrier remains substantially intact until at least 50 % of the virus yield has been released.

METHODS

Virus stocks, virus and ^{32}P assays, media, preparation of chick embryo cells and monolayers, and the one-step growth curves used in the 'loss' experiments were as described by Cooper (1957*a*, *b*).

Measurement of phosphate-impermeable volume (*Table* 1). Ten 20 hr. cell monolayers were washed with warm phosphate buffer saline (PBS) and the cells removed by 10 min. incubation with 0·25 % trypsin in Earle's saline, washed once with cold PBS, chilled thoroughly in ice-water and centrifuged at 500 **g** for 2 min. in a urine-protein centrifuge tube, accurately calibrated to 0·4 ml. by 0·01 ml. intervals and to 1 ml. by 0·1ml. intervals. The pellet volume was *c.* 0·4 ml. The supernatant fluid was sucked off, the remaining volume noted, then 0·5 ml. of cold PBS containing about 0·1 μc ^{32}P (accurately known) and 150 μg. P was added, and the cells thoroughly resuspended by gentle pipetting. A 0·1 ml. sample ('1st sample') was diluted into 1·9 ml. PBS for total ^{32}P and haemocytometer cell counts; the suspension contained single cells free of debris with a few clumps of 2–3 cells. This sample gives the total number of cells in the preparation, and comparison of its ^{32}P content with that known to be added gave the efficiency of mixing (complete within experimental error). Since rate of P exchange did not increase above 0·8 mM (Fig. 2), the use of the higher phosphate concentration of PBS (10 mM) rather than Earle's saline minimized loss of ^{32}P from the supernatant fluid by reciprocal exchange. Any loss was then due to diffusion into the cell; keeping the cells at 0° (see below) decreased any exchange still further. After sampling ('1st sample'), the tube was centrifuged for 2 min. at 500 **g** in an ice-filled cup. A 0·1 ml. sample ('2nd sample') was removed from the supernatant fluid for ^{32}P assay and the pellet volume noted. The cells were resuspended and the whole contents frozen in dry-ice + ethanol mixture and thawed (one cycle), when the cell debris was centrifuged at 1000 **g** for 5 min. and the supernatant was assayed for ^{32}P ('3rd sample'). Finally the sediment was resuspended and a sample was taken for intact cell count ('4th sample'). The experiment was performed with a short (3 min.) and a long (180 min.) interval between addition of ^{32}P and centrifuging.

RESULTS

Temperature dependence of phosphate exchange

A ^{32}P 'loss' experiment (Cooper, 1957*b*) was performed, one set of washed radioactive monolayers overlaid with non-radioactive fluid medium being held at 37° in a CO$_2$ incubator, and another identical set at 3° in a vacuum desiccator filled with a 5 % (v/v) CO$_2$ in air mixture. The rate of ^{32}P release in the cold plates during the first 4 hr. was about 3 % of the rate at 37°, indicating that simple diffusion did not play much part in the release and that phosphate exchange at 0° was likely to be negligible. This was to be expected from the suspected enzymic nature of the exchange, and did not exclude 'exchange adsorption', but its demonstration was relevant for the experiment to be described next.

Phosphate-impermeable volume

Determination of this volume depends upon the changes in extracellular ^{32}P concentration observed when a known amount of ^{32}P in a known volume is added to a cold thick cell suspension, using the procedure described in Methods. The results are given in Table 1, and indicate that under these conditions the phosphate-impermeable volume of $18{\cdot}4 \times 10^7$ cells was 0·19 ml. (3 min. contact), so that the average phosphate-impermeable volume/cell was 1030 μ^3 (radius $= 6{\cdot}26\,\mu$). These are very similar values to those of Dulbecco & Vogt (1954) for the visible dimensions of these cells (mean $= 1180\,\mu^3$, radius $= 6{\cdot}55\,\mu$). The phosphate-impermeable volume is likely to be somewhat less than the total volume of the cells (compare *Micrococcus pyogenes* (*Staphylococcs aureus*), Mitchell, 1953), but it is not legitimate to calculate the depth of the barrier below the visible surface from these figures. The intercellular space is within the expected range for non-uniform non-deformable spherical objects.

Table 1. '*Phosphate-impermeable volumes' of chick embryo cells, measured after* 3 *and* 180 *min. contact with external* $^{32}PO_4$.

Column 3 is calculated on the assumption that all ^{32}P is extracellular, althouth this is probably not so after 180 min. contact. No attempt was made to measure the minimum pellet volume (i.e. hard-packed cells) for fear of damaging the cells.

	Total ^{32}P content of prepn. (cts./min.) (1)	^{32}P content of supernatant (cts./min./ml.) (2)	Therefore vol. extra-cellular fluid 1 ÷ 2 (ml.) (3)	Total vol. of prepn. (ml.) (4)	Therefore P-impermeable vol. (4) − (3) (ml.) (5)	Vol. pellet (ml.) (6)	Therefore inter-cellular space $\left(\frac{(6)-(5)}{(6)}\right)$ (7)	Total No. intact cells (visible count) × 10^7 (8)
3 min. contact								
After 1st sample and centrifuging	3·50 × 10^4	4·80 × 10^4	0·73	0·92	0·19	0·39	0·51	18·4
After 2nd sample, freeze thaw and centrifuging	3·03 × 10^4	3·78 × 10^4	0·80	0·82	*c.* 0·02	0·38	*c.* 0·95	1·2
180 min. contact								
After 1st sample and centrifuging	8·04 × 10^5	10·7 × 10^5	0·75	0·90	0·15	0·39	0·62	28·4
After 2nd sample, freeze thaw and centrifuging	6·97 × 10^5	8·90 × 10^5	0·78	0·80	*c.* 0·02	0·39	*c.* 0·95	3·5

After 180 min. contact at 0° the apparent phosphate-impermeable volume had decreased to $528\mu.^3$/cell, suggesting that phosphate was able to pass the osmotic barrier slowly under these circumstances, a finding supported by the small but significant leakage of ^{32}P at 3° and the slow release of ^{32}P in absence of external phosphate at 37°; thus transfer of phosphate may not be strictly by reciprocal exchange, even in the non-growing cell.

Rupture of the cells, by one cycle of freezing and thawing, immediately (within the centrifuging time of 2–3 min.) diminished the phosphate-impermeable volume to $<10\%$ of the intact volume; the pellet volume of the debris was the same as that of the cells, but the supernatant fluid had become viscous and opalescent.

Phosphate-retaining mechanism

One would think it necessary for cell function that a phosphate-excluding barrier should also be able to retain phosphate already in the cell. That this is so is shown by the relatively slow leakage at 37° in the absence of external phosphate (Figs. 1, 2) and the almost complete retention at 5°, while rupture of the cell by freezing-thawing (Table 4, Cooper, 1957*b*) or by shearing forces

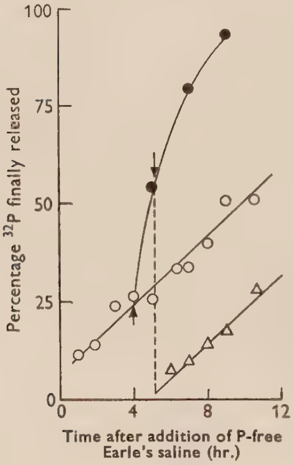

Fig. 1

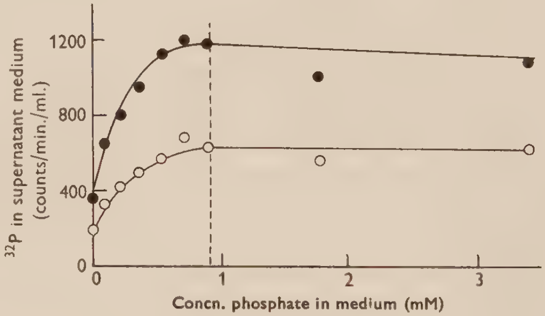

Fig. 2

Fig. 1. Release of ^{32}P from 20 hr. chick embryo cell monolayers grown in labelled medium into phosphate-free Earle's saline (P-free ES). The labelled plates were washed twice with 5 ml. warm complete ES and twice with P-free ES before adding 5 ml. warm P-free ES. To one series of plates (●–● and △–△) sufficient phosphate at pH 7 was added in 0·1 ml. of water after 4 hr. to restore the P to its strength in Earle's saline; half of this series (△–△) had the phosphate-containing ES removed 1 hr. later, was washed twice with warm P-free ES and 5 ml. warm P-free ES substituted. The arrows represent the points of addition and removal respectively of external phosphate.

Fig. 2. Effects of varying the concentration of external phosphate on the rate of ^{32}P release from labelled 20 hr. chick embryo cell monolayers. The monolayers were washed three times with P-free ES, and 5 ml. of warm P-free ES containing varying amounts of added phosphate at pH 7 were added. Samples were removed from the supernatants for ^{32}P assay after 70 (○–○) and 135 min. (●–●). The broken line represents the concentration of phosphate in ES.

at 0° (Table 3*a*, Cooper, 1957*b*) liberated much inorganic phosphate within 2 min. in sucrose-soluble form. In one experiment a 0·02 ml. sample of the sucrose-soluble fraction of frozen-thawed radioactive cells was placed on a filter-paper strip soaked in 0·05 M-sodium citrate buffer (pH 6·0) in parallel with a 0·01 ml. 0·1 M-Na_2HPO_4 sample. Without drying the paper, the ends were placed in electrode compartments containing the buffer, the centre portion being immersed in CCl_4, while a current of 5 mA. was passed at 800 V for 1·5 hr. The strip was then dried and sprayed with the reagent of Bandurski & Axelrod (1951) and irradiated with ultraviolet light to develop the blue-green colour characteristic of inorganic phosphate. The orthophosphates produced blue-green spots 22 cm. from the origin which coincided with a peak containing 85 % of the radioactivity of the preparation and 90–100 % of the acid-soluble inorganic ^{32}P; under these conditions the mobility of the fastest monophosphate, uridylic acid, would be 60 % of the orthophosphate. There was no 'streaking' of the radioactivity which would indicate decomposition on the paper.

This procedure therefore separated a large proportion of the cellular ^{32}P as a fraction with the same mobility as inorganic orthophosphate. The mildness of the extraction and separation methods used strongly suggests that it is in this form in the intact cell, and the quantitative identity of the orthophosphate with the 'acid-soluble inorganic phosphate' fraction, where other ^{32}P fractions are much smaller, suggests that these fractions are in fact identical. It therefore seems likely, although not proven, that the external phosphate is in reciprocal exchange with an internal pool of free phosphate ions.

Effect of virus infection on the rate of ^{32}P *exchange*

Previous results (Cooper, 1957*b*) showed that, unlike bacteriophage (Puck & Lee, 1954) vesicular stomatitis virus infection had no effect on rate of ^{32}P release, if the commencement of release corresponded to the initiation of infection, so that the effective period examined was the first half of the latent period. Fig. 3 shows the first hr. in more detail, and also that when the replacement of radioactive by non-radioactive medium was delayed so that the most rapid release of ^{32}P extended over successively later periods, the lack of viral effect on rate of ^{32}P release could be demonstrated up to the time that exponential virus release was almost complete; a systematic difference in ^{32}P release rate of 10 % should be detectable.

The characteristics of the exchange

Assuming for *Micrococcus pyogenes* (*Staphylococcus aureus*) that the cell-wall transport of phosphate was of the exchange-diffusion type, and that the internal and external pools of inorganic phosphate were homogeneous, Mitchell (1953) derived the theoretical relationship $\ln\left[\frac{1-R_0}{1-R_t}\right]=kt$ for the process of equilibration during gain or loss experiments. R_0 and R_t were the 'relative specific activities' of either I, the internal phosphate (i.e. cellular acid-soluble inorganic phosphate) for 'gain' experiments, or E, the external phosphate for 'loss' experiments at the beginning of the experiments ($t=0$) and time $=t$, respectively.

$$R_t = \frac{^{32}\mathrm{P}_t \times K}{\mathrm{P}},$$

where $^{32}\mathrm{P}_t$ = total $^{32}\mathrm{P}$ content of E or I at time $=t$ and P = total phosphate of E or I (constant throughout the experiment). K is defined by the relationship (equation A) which exists at complete equilibration:

$$\frac{^{32}\mathrm{P}_\infty \times K}{\mathrm{P}} = R_\infty. \qquad \text{Equation (A)}$$

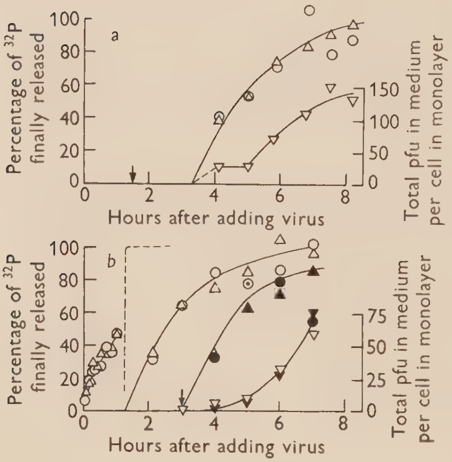

Fig. 3

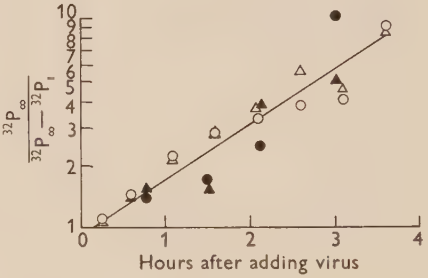

Fig. 4

Fig. 3. Effect of one-step vesicular stomatitis virus infection on the rate of ^{32}P exchange from labelled 20 hr. chick embryo cell monolayers. Different portions of the virus release curves are covered by the most rapid periods of ^{32}P release. The labelled medium was removed, virus diluted in 0·5 ml. of the labelled medium was added so as to give average multiplicities of adsorption (M) of 6 (*a*) and 2 (*b*); after 30 min. 4·5 ml. of the labelled medium was replaced. At various times indicated by the commencement of the release curves after the addition of virus (1·2 and 3·0 hr., for M = 2, and 3·3 hr. for M = 6) the medium was removed, the monolayers washed rapidly twice with warm ES + 4 % ox serum (ES/Ox) and 5 ml. non-radioactive ES/Ox added. In one experiment (0–1 hr., (*b*)) virus was added to the washed monolayers in 5 ml. ES/Ox at the same concentration (10^8/ml.) as the other curves of (*b*), so that the multiplicity of adsorption within 30 min. should be about the same (*c*. 2), and samples were removed at close intervals to examine in detail the period of adsorption (0–1 hr.). The broken vertical line represents the rate of ^{32}P release from ruptured cells. (○ and ●) = control, and (△ and ▲) = virus-treated monolayers; (▽ and ▼) = virus release curves. The arrows indicate the end of the latent period (release of 1 pfu/cell) calculated from logarithmic plots.

Fig. 4. Relationship between t and inorganic orthophosphate ^{32}P in external environment for 'loss' experiments, or in internal environment for 'gain' experiments, after equilibration was complete ($^{32}\mathrm{P}_\infty$), and at time $=t$ ($^{32}\mathrm{P}_t$), for the exchange of phosphate across the chick cell osmotic barrier at pH 7 and 37°. Circles = control and triangles = virus-infected monolayers; open points = ^{32}P moving into the cells (gain), filled-in points = ^{32}P moving out of the cells (loss). The data is that of figs. 2 and 3 (Cooper, 1957*b*); the latent period ended at 3·7 hr. Commencement of equilibration coincided with addition of virus for the loss experiment, but was 0·15 hr. after virus for the gain experiment.

R_∞ is defined as $=1$, and $^{32}\mathrm{P}_\infty$ as $=$amount of ^{32}P present in E or I in loss or gain experiments when equilibration was complete. Thus the 'relative specific activities' indicate the completeness of equilibration. Mitchell's equation depended on the demonstration that there was negligible net change of P during the period of exchange, as was verified experimentally.

The data of Cooper (1957*b*) indicate that synthesis of organic phosphate in the chick-cell culture system was slow compared with the exchange, and that there was little net change in P during gain or loss experiments. Using the same arguments and definitions, Mitchell's equation can be adapted to the data available from either the loss or gain experiments with chick embryo cells as follows:

At $t=0$, $R_0=0$, therefore

$$\ln\left[\frac{1-R_0}{1-R_t}\right]=\ln\left[\frac{1}{1-{}^{32}P_tK/\mathrm{P}}\right]=\ln\left[\frac{\mathrm{P}/K}{\mathrm{P}/K-{}^{32}\mathrm{P}_t}\right]=kt.$$

Substituting $^{32}\mathrm{P}_\infty/R_\infty$ for P/K (from equation A):

$$\ln\left[\frac{^{32}\mathrm{P}_\infty}{^{32}\mathrm{P}_\infty-{}^{32}\mathrm{P}_t}\right]=kt. \qquad \text{Equation (B)}$$

A plot of $\log_{10}\left[\frac{^{32}\mathrm{P}_\infty}{^{32}\mathrm{P}_\infty-{}^{32}\mathrm{P}_t}\right]$ against time for both the gain and loss experiments presented in figs. 2 and 3 of Cooper (1957*b*) (Fig. 4), and also for the gain experiments in Fig. 3 above, showed an approximately linear relationship, suggesting a similar mechanism in chick cells to that operating in *Micrococcus pyogenes*. No regular difference was noticeable between infected and control points.

DISCUSSION

It is felt that no mechanisms other than a phosphate-restrictive cell wall can explain the existence of an apparent phosphate-impermeable volume nearly coincident with that of the cell volume, as the specific volume of the cell constituents (as measured by the phosphate-impermeable volume after rupture) is less than 10% of the cell volume. The very rapid breakdown of this restriction on cell-rupture, together with the close correlation between radioactivity and inorganic orthophosphate after very mild separation, indicates that either the phosphate linkages with any enzymically formed intracellular matrix are unusually labile or that the cell holds its reservoir of orthophosphate in the form of free ions retained by a restrictive cell wall.

It is interesting to discuss the nature of the restriction to phosphate, that is, of the mechanism by which phosphate is allowed to pass the cell wall. It has been pointed out elsewhere (Cooper, 1957*b*) that the 'reversible' acid-soluble inorganic phosphate behaves as a pool which exchanges with the inorganic phosphate of the medium in a largely reciprocal fashion; with a total cell volume of $1180\,\mu^3$ and $2{\cdot}5\times10^{-7}\,\mu$g. P/cell its concentration is at least 6·8 mM or 7 times that of the medium. It has been possible to set up two hypothetical models to explain the way in which this phosphate is concentrated and retained. One was that the cell wall is impermeable to phosphate in the physical sense, retaining as in a bag free phosphate ions whose entry requires a specific transport mechanism, and the other that phosphate diffuses physically into the cell at least as rapidly as the exchange process and is then either immobilized in a phosphate-accepting framework by a chemical process, probably enzymic, or retained by Donnan-type equilibria. These two hypotheses were compared above with 'exchange-diffusion' and 'exchange-adsorption' as defined by Mitchell (1953) for *Micrococcus pyogenes* (*Staphylococcus aureus*). The fact that the cell appears impermeable to phosphate after only 3 min. of contact with phosphate merely means that any inward physical diffusion is too slow to be measureable within this time. An exponential process 50 % complete in 70 min. would be only 3 % complete in 3 min. If one can therefore demonstrate, under conditions where any enzymic step is negligible, that the rate of diffusion into or out of the cell at 37° is slower than the exchange rate at 37°, exchange adsorption as defined above is excluded. It is shown above that liberation of phosphate from the cell is negligible at 0°, and it is mentioned in an appendix that physical diffusion inwards may not predominate at 37°, although more data is required to be certain of this point. These points would therefore favour exchange diffusion.

It is shown above that the kinetics of the exchange are very similar to those of *Micrococcus pyogenes* (*Staphylococcus aureus*), which can be justified theoretically from consideration of the exchange-diffusion mechanism. It is more than likely that the proportion of the 'exchange' apparently due to physical diffusion is a result of some damage to the cell surfaces during handling. It is interesting that the rate of exchange of phosphate is maximal at about physiological concentrations (Fig. 2); the 50 % equilibration time is 70–90 min. Addition of external phosphate to P-free ES at once initiated the exchange (Fig. 1), and subsequent removal allowed only the slow release again.

Having shown that the relatively slow exchange of phosphate with the medium (compared with the much more rapid release or entry after cell rupture) is due to a restriction at the cell surface. Whatever the nature of this restriction, the lack of effect of VS virus infection on this exchange makes it possible to say that both penetration and release of this virus can result in only a very limited lysis of the cell wall. At all events, release is certainly not accompanied by a 'burst' in the sense in which the term is applied to the release of bacteriophage. This conclusion has frequently been drawn from other types of observations with other viruses (Dulbecco, 1955). Similarly, the fact that ^{32}P release is not retarded by the virus shows that any enzymically-dependent transport is not grossly damaged by passage of the virus.

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REFERENCES

Bandurski, R. S. & Axelrod, B. (1951). The chromatographic identification of some biologically important phosphate esters. *J. biol. Chem.* **193**, 405.

Cooper, P. D. (1957*a*). Some characteristics of vesicular stomatitis virus growth curves in tissue culture. *J. gen. Microbiol.* **17**, 327.

Cooper, P. D. (1957*b*). Paths of phosphate transfer in normal chick embryo cells and in cells infected with vesicular stomatitis virus. *J. gen. Microbiol.* **17**, 335.

Dulbecco, R. (1955). Interactions of viruses and animal cells. A study of facts and interpretations. *Physiol. Rev.* **35**, 301.

Dulbecco, R. & Vogt, M. (1954). One-step growth curve of western equine encephalomyelitis virus on chicken embryo cells grown in vitro and analysis of virus yields from single cells. *J. exp. Med.* **99**, 183.

Greenberg, D. M. (1952). The acid-soluble phosphates in animal metabolism. *Phosphorus metabolism*, vol. II, p. 3. A symposium. Ed. W. D. McElroy and B. Glass. Baltimore: Johns Hopkins Press.

Kamen, M. D. & Spiegelman, S. (1948). Studies on the phosphate metabolism of some unicellular organisms. *Cold Spr. Harb. Symp. quant. Biol.* **13**, 151.

Mitchell, P. (1953). Transport of phosphate across the surface of *M. pyogenes*: nature of the cell 'inorganic phosphate'. *J. gen. Microbiol.* **9**, 273.

Mitchell, P. & Moyle, J. (1953). Paths of phosphate transfer in *M. pyogenes*: phosphate turnover in nucleic acids and other fractions. *J. gen. Microbiol.* **9**, 257.

Mitchell, P. & Moyle, J. (1956). Osmotic function and structure in bacteria. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 150.

Puck, T. T. & Lee, H. H. (1954). Mechanism of cell wall penetration by viruses. I. An increase in host cell permeability induced by bacteriophage infection. *J. exp. Med.* **99**, 481.

Sacks, J. (1948). Mechanism of phosphate transfer across cell membranes. *Cold Spr. Harb. Symp. quant. Biol.* **13**, 180.

APPENDIX

An approximation to the kinetics of the entry of phosphate into the cell at 0° can be obtained from the 'phosphate-impermeable volume' data after 3 hr. at 0° (Table 1). If the same principles apply to a process of reversible diffusion at 0° as to the exchange at 37°, the relationship

$$\ln\left[\frac{{}^{32}\mathrm{P}_\infty}{{}^{32}\mathrm{P}_\infty - {}^{32}\mathrm{P}_t}\right] = k_0 t$$

will also hold. Presumably the 'phosphate-impermeable volume' does not actually shrink, but rather the data indicates a progressive diffusion of phosphate from the external space (volume $= V_e$ ml.) to the internal (V_i ml., = phosphate impermeable volume at $t=0$). It is further assumed that the cell's internal volume remains constant during the experiment. Then ${}^{32}\mathrm{P}_\infty$, the ${}^{32}\mathrm{P}$ content of the internal space at complete equilibration, $= \dfrac{T.\,V_i}{V_e + V_i}$, where $T =$ total ${}^{32}\mathrm{P}$ content of the system, since then the internal and external concentrations should be approximately equal (the internal total inorganic phosphate, at *c.* 7 mM, is of the same order as the external phosphate (10 mM) in this experiment).

In Table 1, the 'phosphate-impermeable volume' $= c.\ \frac{1}{2}V_i$ after 3 hr. The external concentration of ^{32}P is then $= \frac{T}{V_e + \frac{1}{2}V_i}$ and the amount of ^{32}P in the external space $= \frac{T.\,V_e}{V_e + \frac{1}{2}V_i}$.

Therefore the internal $^{32}\mathrm{P}_t = T - \left[\frac{T.V_e}{V_e + \frac{1}{2}V_i}\right] = \frac{T.V_i}{2V_e + V_i}$.

Therefore

$$\frac{^{32}\mathrm{P}_\infty}{^{32}\mathrm{P}_\infty - {}^{32}\mathrm{P}_t} = \frac{1}{1 - \left[\frac{V_e + V_i}{2V_e + V_i}\right]} = 2 + \frac{V_i}{V_e}.$$

Therefore

$$\ln\left[2 + \frac{V_i}{V_e}\right] = kt \text{ (from equation B).} \qquad \text{(Equation C)}$$

where $t' =$ time at which the phosphate impermeable volume $= \frac{1}{2}V_i$.

In the exchange experiments of Fig. 4, V_i/V_e is very small and $t' = 1{\cdot}2$ hr.; the value of $\frac{^{32}\mathrm{P}_\infty}{^{32}\mathrm{P}_\infty - {}^{32}\mathrm{P}_t} = 2$ gives a 50 % equilibration time of 70–90 min., which in this case therefore coincides with the time at which the phosphate-impermeable volume appears to be $= \frac{1}{2}V_i$. From equation (B), $k_{37} = 0{\cdot}58$ hr.$^{-1}$. V_i/V_e is larger in the experiment of Table 1 (*c.* 0·2), giving, on the basis of the single point at 3 hr., where the phosphate-impermeable volume happens to be $\frac{1}{2}V_i$, a value for k_0 for the entry of phosphate at 0° of 0·26 hr.$^{-1}$, or approximately one-half that of the k value for entry at 37° (Equation C). Thus the effect of temperature appears considerably greater than one would expect from a purely physical mode of entry, suggesting that as for 'loss' the physical process is not predominant at 37°.

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Parle, J. N. (1957). *J. gen. Microbiol.* 17, 363–367

Yeasts Isolated from the Mammalian Alimentary Tract

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SUMMARY: A survey of the yeasts found in the alimentary canal of 169 animals has been carried out. Representatives of 17 species and 6 genera were isolated but of these only 4 species, *Torulopsis pintolopesii*, *Saccharomycopsis guttulata*, *Candida albicans*, and *T. glabrata* were considered to be true intestinal types. *C. parapsilosis* and *C. krusei* were classed as intermediates and the remainder as transients. The validity of the species *T. pintolopesii* is considered doubtful.

Although the yeast flora of the human alimentary tract has been studied to some extent little is known of the yeasts which occur in other animals. A few early investigations such as those of Catanei (1925) on monkeys and Talice (1932) on hedgehogs established the presence of members of the genus *Candida* in the intestines of healthy animals. De Mello (1940) reported the presence of *Candida* and *Geotrichoides* spp. in the intestines of normal laboratory animals. Coutelon & Cochet (1942) also investigated laboratory animals but reported only those species of medical interest from the yeasts isolated. In a survey of fungi isolated from suspected mycotic infections of domestic animals Ainsworth & Austwick (1955) noted the importance of the yeast component in the mycoflora of the alimentary canal and respiratory organs. The present survey covers a somewhat wider range of animals than those previously noted.

METHODS

Samples were taken from 169 animals: 14 cows, 30 rabbits, 15 sheep, 10 guinea pigs, 5 opossums, 10 monkeys, 15 cats, 10 dogs, 10 hedgehogs, 15 mice, 20 pigs and 15 rats.

Sampling and cultivation techniques

Wherever possible the animals were sacrificed and samples were taken from the stomach, small intestine and large intestine. Appropriate sections of the alimentary tract were removed aseptically and their contents expressed into sterile Petri plates. Material from each section was inoculated on to the surface of Sabouraud agar (pH 4·0) and also into glucose yeast-extract broth (pH 4·0). In cases where the animal could not be killed samples of fresh faeces were cultured by inoculating material from the centre of the specimen on to Sabouraud agar and also into glucose yeast-extract broth.

Sabouraud agar proved unsatisfactory for the isolation and growth of *Torulopsis pintolopesii* van Uden, a fastidious yeast found in rats and mice, and a medium containing liver extract was used to isolate and maintain this species. Glucose yeast-extract broth at pH 4·0 was completely satisfactory for the primary isolation of all species of yeasts found during this survey, the low pH value and restricted nitrogen source diminishing bacterial growth to a considerable extent. All cultures were incubated at 37° to exclude species unable to grow at the body temperature of the animals examined, and were kept for a week before being discarded as negative.

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The methods proposed by Lodder & Kreger-van Rij (1952) were used throughout this survey for the identification and classification of the yeasts isolated. Methods for the identification of *Saccharomycopsis guttulata* were based on morphology and ecology, the organisms are large and vacuolated and have a distinctive growth form. Ascospores, which were produced on Gorodkowa agar, are oval and up to three are formed per ascus. Ecologically the species is restricted almost entirely to the rabbit. An investigation of some of the physiological properties of *S. guttulata* was carried out (Parle, 1956).

RESULTS

Yeasts were isolated from 107 of the animals sampled and members of 6 genera and 17 species were obtained. In certain animals dominant yeast species appeared. This was particularly marked in the case of two unusual types: *Saccharomycopsis guttulata* which occurred in all the rabbits and *Torulopsis pintolopesii* which was found in all the guinea pigs, 73 % of the mice and 66 % of the rats examined. *Candida albicans* was the dominant species found in hedgehogs, sheep and opossums and was the most widely distributed of the species isolated. The remaining yeasts appeared to be rather sporadic in their distribution and many species were isolated only once or twice throughout the survey.

DISCUSSION

Yeasts isolated from the alimentary canal may be divided into two classes, those belonging to the true intestinal flora and those which are merely transitory in nature being ingested with the food. The physiology and distribution of the yeasts isolated during the survey were the criteria used to distinguish between these two groups. Only yeasts which require environmental conditions, including special growth factors, similar to those found in the intestine, or yeasts which occurred commonly in the intestine but have rarely if ever been reported outside it, were considered to be true intestinal types. Yeast species which are commonly found in situations other than the alimentary canal were considered to be transients. A division based on these criteria must necessarily have somewhat indefinite boundaries but in the present state of knowledge no other system of classification appears to be any more satisfactory. Of the 17 species isolated from the animals examined, only four, *Saccharomycopsis guttulata*, *Torulopsis pintolopesii*, *T. glabrata* and *Candida albicans* were considered to belong to the true intestinal flora. *C. parapsilosis* and *C. krusei* may also belong to this group but their position is rather doubtful.

Table 1. *Animals sampled and species of yeasts isolated*

	Cow	Guinea pig	Opossum	Rabbit	Sheep	Monkey	Cat	Dog	Hedgehog	Mouse	Pig	Rat	Total
Number sampled	14	10	5	30	15	10	15	10	10	15	20	15	169
Number positive	6	10	3	30	15	8	—	7	10	11	10	10	120
Candida albicans (Robin). Berkhout. Lodder & Kreger-van Rij (1952), p. 466, figs. 170–2	.	.	3	.	8	3	.	2	9	.	1	.	.
C. krusei (Castellani). Berkhout. Lodder & Kreger-van Rij (1952), p. 492, figs. 178–9	.	.	.	.	1	3	.	.	1	.	5	.	.
C. mycoderma (Reess) Nov. comb. Lodder & Kreger-van Rij (1952), p. 477, figs. 173–4	.	.	.	.	.	.	.	1	.	.	.	.	.
C. parapsilosis (Ashf.) Lang. et Talice. Lodder & Kreger-van Rij (1952), p. 553, figs. 209–210.	.	.	.	5	2	.	.	.	.	.	.	.	.
C. tropicalis (Caste.) Berkhout. Lodder & Kreger-van Rij (1952), p. 502, figs. 182–3	.	.	.	.	2	.	.	.	.	.	.	.	.
C. utilis (Henneberg) nov.comb. Lodder & Kreger-van Rij (1952), p. 546, figs. 205–6	.	.	.	.	.	.	.	.	.	1	.	.	.
Torulopsis famata (Harrison) nov.comb. Lodder & Kreger-van Rij (1952), p. 417, fig. 147	.	.	.	.	.	.	.	.	1	.	.	.	.
T. glabrata (Anderson) Lodder et De Vries. Lodder & Kreger-van Rij (1952), p. 407, fig. 142	.	.	.	.	.	1	.	.	.	.	.	.	.
T. pintolopesii (van Uden), van Uden (1952)	.	10	.	.	.	.	.	.	.	11	.	10	.
Trichosporum capitatum (Diddens & Lodder). Lodder & Kreger-van Rij (1952), p. 634, figs. 255–6	.	.	.	.	.	.	.	1	.	.	.	.	.
T. cutaneum (de Beurm., Gougerot & Vaucher) Ota. Lodder & Kreger-van Rij (1952), p. 169, figs. 246–7	.	.	.	.	.	.	.	.	.	1	4	.	.
T. pullulans (Lindner) Diddens et Lodder. Lodder & Kreger-van Rij (1952), p. 615, figs. 244–5	3	.	.	.	.	.	.	.	.	.	.	.	.
Rhodotorula minuta (Saito). Harrison. Lodder & Kreger-van Rij (1952), p. 663, fig. 267.	.	.	.	.	.	.	.	.	1	.	.	.	.
R. mucilaginosa (Jorgensen) Harrison. Lodder & Kreger-van Rij (1952), p. 655, fig. 264.	.	.	.	3	.	1	.	.	1	.	.	.	.
Hansenula anomala (Hansen) H. et P. Sydow. Lodder & Kreger-van Rij (1952), p. 251, figs. 73–5	3	.	.	.	.	.	.	.	.	.	.	.	.
Saccharomyces cerevisiae Hansen. Lodder & Kreger-van Rij (1952), p. 123, figs. 22–3	.	.	.	.	.	.	.	1	.	.	.	.	.
Saccharomycopsis guttulata (Robin) Schiönning. Lodder & Kreger-van Rij (1952), p. 299, figs. 100–101	.	.	.	.	30	.	.	.	.	.	.	.	.

Saccharomycopsis guttulata. This yeast is extremely specialized in both its habitat and growth requirements. It is found in the intestines of rabbits and occasionally in guinea pigs (Lodder & Kreger-van Rij, 1952). Microscopic examination of the intestinal contents showed a 100 % incidence in the rabbits sampled, although it was not found in any of the other animals studied. Considerable difficulty was experienced in attempts to isolate *S. guttulata* and identification depended on its microscopic appearance.

Torulopsis glabrata. Only three strains of this organism were isolated, but since it has rarely been found except in association with the animal body it was considered to belong to the true intestinal flora.

Torulopsis pintolopesii. This yeast was first reported by van Uden (1952), who isolated it from the livers of sick mice. Rats and mice of wild and of laboratory varieties, and guinea pigs, seem to harbour this yeast. Samples of food and litter from cages containing animals carrying *T. pintolopesii* were examined but the organism was not isolated except from faeces or intestinal contents. The limited distribution of this organism may be due to two factors, the restricted temperature range (18°–40°) over which growth will occur, and its requirement for some as yet unidentified growth factor. The validity of the species *T. pintolopesii* seems somewhat doubtful since except for its unusual growth factor and temperature requirements the organism is indistinguishable from *T. glabrata.* In a group as phylogenetically heterogeneous as the Torulopsidae the requirement for a specific growth factor does not seem reason enough to establish a new species especially as some strains of *T. glabrata* are difficult to grow on common laboratory media. Further investigation of this problem may indicate that *T. pintolopesii* should be classed as a variant of *T. glabrata* rather than as a separate species.

Candida albicans. This yeast occurs widely in animals and has been reported in humans by a number of workers (Benham & Hopkins, 1933; Marples & di Menna, 1952; Bezjak, 1954), in rats, rabbits, hens and pigeons by Coutelon & Cochet (1942), domestic birds, dogs and cattle by Ainsworth & Austwick (1955), and the European hedgehog by Talice (1932). Because of its wide distribution in the intestines of animals there must be an almost constant inoculation of the soil with this yeast. However, although several workers have investigated soils *C. albicans* has been isolated with certainty on only three occasions from this source, by di Menna (1955). Such a distribution is of some interest ecologically since *C. albicans* unlike *Saccharomycopsis guttulata* and *Torulopsis pintolopesii* is not fastidious in its requirements. It is possible that the antibiotics which are produced by a number of soil fungi and which are known to be active against *C. albicans* are responsible for its rather sporadic occurrence in soil.

Candida parapsilosis and *C. krusei.* These have been isolated from the animal body both as saprophytes and possible parasites on numerous occasions but they also occur commonly in soil and on vegetation. Because of the methods used here to divide the yeasts into true or transitory flora these species occupy an intermediate position in this classification and must continue to do so until more is known of the role of yeasts in the alimentary canal.

All members of the remaining eleven species isolated from various animals were classed as transients and probably entered with the food.

A strain of *Torulopsis pintolopesii* has been deposited with the Centraalbureau voor Schimmelcultures (Yeast Division), Delft, Netherlands.

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REFERENCES

AINSWORTH, G. C. & AUSTWICK, P. K. C. (1955). A survey of animal mycoses in Britain. *Trans. Brit. mycol. Soc.* 38, 376.

BENHAM, R. W. & HOPKINS, A. M. (1933). Yeast-like fungi found on skin and in the intestines of normal subjects. *Arch. Derm. Syph., N.Y.* 28, 532.

BEZJAK, V. (1954). Incidence of yeast-like fungi in the oral cavity. *Acta Medica iugosl*, VIII, **2**, 200.

CATANEI, A. (1925). Presence d'un Monilia sur la langue de singes d'Algerie. *C.R. Soc. Biol., Paris*, **93**, 92.

COUTELON, F. & COCHET, G. (1942). Les rongeurs domestiques, reservoirs de virus en mycopathologie humaine et veterinaire. *Ann. Parasit. hum. comp.* **19**, 85.

LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts.* Amsterdam: North Holland Publishing Company.

MARPLES, M. J. & DI MENNA, M. E. (1952). Incidence of *Candida albicans* in Dunedin New Zealand. *J. Path. Bact.* **64**, 497.

MELLO, I. F. DE (1940). A report on the characters and identification of the yeasts living in commensalism in the intestine of some laboratory animals. *Proc. Indian Acad. Sci.* (Sect. B), **12**, 17.

MENNA, M. E. DI (1955). A search for pathogenic species of yeasts in New Zealand soils. *J. gen. Microbiol.* **12**, 54.

PARLE, J. N. (1956). The growth of *Saccharomycopsis guttulata. Leeuwenhoek ned. Tijdschr.* **22**, 237.

TALICE, R. V. (1932). Parasitisme des Herissons par les Mycotorulees. *Ann. Parasit. hum. comp.* **10**, 81.

UDEN, N. VAN (1952). Zur Kenntnis von *Torulopsis pintolopesii* sp.nov. mit Beobachtungen über die parasitäre Phase von *Acladium castellanii* Pinoy. *Arch. Microbiol.* **17**, 199.

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WATSON, S. W. & RAPER, K. B. (1957). *J. gen. Microbiol.* **17**, 368–377

Labyrinthula minuta sp.nov.

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SUMMARY: A new marine species of *Labyrinthula* is described for which the name *L. minuta* is proposed because of its diminutive proportions. This species has been isolated repeatedly from *Ulva lactuca* and other algae collected from the Atlantic Ocean in the region of Woods Hole, Massachusetts. The vegetative stage, as in other species of *Labyrinthula*, consists of independent cells interconnected by threads of slime which form a net-like structure called the net-plasmodium. *L. minuta* differs from all previously described species of this genus in the size, shape, and movement of its cells, in its method of cell division, its colonial morphology and by the formation in aged cultures of multinucleate bodies which resemble miniature plasmodia. No fructification has yet been observed. This species has been grown in pure reproducible laboratory culture on a serum + sea water medium for a period of five years.

The genus *Labyrinthula* is composed of unicellular, aquatic micro-organisms that are characterized by a gliding motility on self-formed slime filaments. Extension, branching, and anastomosis of such filaments connect the cells together into a colony (Pl. 1, figs. 1, 2). Such a colony, when viewed under low magnification on an agar surface, presents a view of a finely meshed network often called a net-plasmodium (Zopf, 1892). The unique mobility of the cells restricted to the pre-formed slime tracts distinguishes *Labyrinthula* from all other known genera in the plant and animal kingdoms.

Cienkowski (1867) established the genus *Labyrinthula* with the description of two marine species, *L. vitellina* and *L. macrocystis*. Following this, two freshwater species were recorded as *L. cienkowskii* by Zopf (1892) and *L. zopfii* by Dangeard (1910). Later, Valkanov (1929) assigned the name *L. zopfii* to a marine form, not realizing that Dangeard had already applied this name to a freshwater species. In 1932 Dangeard reported another marine species as *L. chattonii*. Upon reviewing the reports of these six species, Young (1943) proposed a consolidation into three species and two varieties, and suggested that 'further investigations might even reduce these to a single species'.

Inadequate and possibly inaccurate descriptions exist for two additional organisms which probably belong to this genus, namely: *Labyrinthomyxa sauvageaui* Duboscq (1921) and *Chlamydomyxa labyrinthuloides* Archer (1875). Additionally, *Pseudoplasmodium aurantiacum*, described by Molisch (1926) as a new genus of the Acrasieae, almost certainly represents a species of *Labyrinthula*.

The conflicting and often fragmentary nature of the published reports on *Labyrinthula* is believed to result in substantial measure from the impure and non-reproducible laboratory cultures studied by earlier investigators. Watson & Ordal (1957) recently developed techniques for obtaining pure cultures of *Labyrinthula*, and *L. minuta* was isolated and cultivated on serum + sea water agar by application of the methods described by them. This species differs from all other described members of the genus in one or more of the following characteristics: cell size, cell shape, cell movement, mode of division, formation of a multinucleate stage, and colonial morphology. These distinguishing characteristics have remained constant with each periodic transfer of laboratory cultures maintained for the past five years. The binominal *L. minuta* is applied to the new species because of the diminutive proportions of its cellular elements.

TECHNICAL DESCRIPTION

Labyrinthula minuta sp.nov.

Status vegetalis in agare a sero sanguine aquaque marina parato est unicellularis, calore camerali. Forma ovalis est aut discoidalis, 5–10μ. longitudine, 3–5μ. latitudine. Se findit in tetrades per divisiones seriales angulis rectis. Colore vitreus aut pallido-fulvus. Nucleus est sphaeroideus, 1·5–2·0μ. diametro; est unus nucleolus centralis. E cellis progreditur rete filamentorum inter se connexorum 0·5–1·0μ. latitudine; quibus utentes cellae exhibent mobilitatem labilem, saepe regredientem. E cellis vegetalibus filamentisque constat plasmodium retale seu colonia; ad eius limbum nascuntur cellae multinucleatae post septimanam, 8–22μ. diamentro, singulae habentes 4–64 nucleos. Se frangunt tandem in cellas uninucleatas vegetales; coloniae saepe rubo-fulvae post tres septimanas aut plus. Sori generatio non visa est.

Hab. Seclusa ab *Ulva* sp. aliisque algis ex oceano Atlantico carptis, Woods Hole, Massachusetts, U.S.A., 1952.

Vegetative stage on serum + sea water agar at room temperature unicellular, oval to discoidal, 5–10μ. by 3–5μ., dividing into tetrads by successive division at right angles, colourless to pale orange; nucleus spherical, 1·5–2·0μ. in diam. with one central nucleolus; cell producing a net-work of interconnected filaments 0·5–1μ. wide on which these cells exhibit gliding motility, often reversible, the vegetative cells and filaments constituting the net-plasmodium or colony; multinucleate bodies appear at the periphery of the colony after 1 week, 8–22μ. diameter, 4–64 nuclei per cell, eventually fragmenting into uninucleate vegetative cells; colonies often reddish orange after 3 weeks or more; vegetative stage in broth culture as on solid agar medium except that cells form micro- or macroscopic tufts which may adhere on the glass surface or be suspended in the liquid medium, such tufts appearing orange or colourless. Sori are not generally observed.

Isolated from *Ulva* sp. and other marine plants collected from the Atlantic Ocean, Woods Hole, Massachusetts, 1952.

ISOLATION AND CULTIVATION

The initial isolation of *Labyrinthula minuta* was made from a fragment of green alga, *Ulva* sp., collected from the undersurface of a landing float at the Woods Hole Oceanographic Institution during the winter of 1952. The following 'hosts' in the Woods Hole region consistently provided us with additional sources of *L. minuta*; the green algae, *Chaetomorpha linium*, *Ulva lactuca*, *Bryopsis*, *plumosa*, *Ectocarpus* sp.; the red algae *Gracilaria confervoides* and *Ceramium rubrum*; and the flowering plant *Zostera marina*. We failed to obtain it from *Zostera marina* from six other areas on the Atlantic seaboard, namely: Prince Edward Island, Canada; New Haven and Milford, Connecticut; Gloucester, Massachusetts; Long Island, New York; Port Erin, Isle of Man. Shores of the Pacific Ocean in North-western United States have not yielded a single specimen of *L. minuta*. However, other species of *Labyrinthula* have been recovered repeatedly from the same area during the past five years.

The technique of Watson & Ordal (1957) was employed for the isolation and cultivation, first in mixed culture and later as a pure culture, of the organism currently described. Any of the following media maintained adequate growth of *Labyrinthula minuta*: (1) 5–10% (v/v) serum in sea water solidified by 0·9% agar, or kept liquid in 0·1% agar; (2) a liquid, partially defined medium described by Vishniac & Watson (1953); (3) a liquid defined medium formulated by Vishniac (1955).

Laybrinthula minuta grows most rapidly at 20–25° but it can be cultivated at a maximum temperature of 35° and a minimum of 10°. For stock cultures, 15° has proved most reliable. All microscopic observations have been made at room temperatures (21–28°).

COLONIAL MORPHOLOGY

The vegetative colony of *Labyrinthula minuta* is composed of individual cells interconnected by their hyaline mucoid filaments (Pl. 1, fig. 7), forming a net-plasmodium. However, this network is demonstrable only with magnifications of ×400 or higher, and when viewed with lower magnifications the cells appear to be scattered at random on the agar surface (Pl. 1, fig. 1). In contrast, the net-plasmodium in other species of *Labyrinthula* can be observed easily with much lower magnifications (Pl. 1, fig. 2). Two characteristics account for this striking difference, namely: the loose arrangement of the cells on the tracks in *L. minuta* and the extreme delicacy of the mucoid filaments formed by this species. (1) The cells usually exist in small clusters of four to eight, or as single cells, separated by distances of 10–100μ. on the connecting filaments. Rarely are the cells lined up on the tracks so that each cell is immediately adjacent to and touching the cell in front and behind it. In all forms of *Labyrinthula* the very thin tracks are too hyaline to be observed under very low magnifications. However, in the larger and earlier described forms the colony appears as a network even under low magnifications because the cells are closely queued and aligned abreast of each other on the tracks (Pl. 2, fig. 8). (2) The filaments of *L. minuta* are only 1–2μ. wide (Pl. 1, fig. 7), and the cells travel only in single file on these thin filaments. Although extremely fine filaments are also found in other forms of *Labyrinthula*, broad filaments up to 50μ. wide are commonly observed, and upon these pathways (Pl. 2, figs. 8, 9) 2–20 cells are frequently seen advancing abreast of one another.

CELLULAR MORPHOLOGY AND MOTILITY

Cell size

The development of relatively simple methods for isolating and propagating *Labyrinthula* in pure culture (Watson & Ordal, 1957) has enabled us to compare *L. minuta* with many strains representing other marine species of this genus. An isolate of *Labyrinthula* believed to represent *L. vitellina* Cienk. is used here for photographic comparison because it is the best defined of all existing species. Except for cell size, the six species previously described would appear to be indistinguishable in the vegetative stage. These species, together with the relative sizes of the vegetative cells, as reported by the describers and subsequent investigators, are presented in Table 1. The only species whose cell dimensions approximate to those of *L. minuta* are *Labyrinthomyxa sauvageaui* Duboscq, *Pseudoplasmodium aurantiacum* Molisch, and *Labyrinthula zopfii* Valk. Upon the basis of size alone, a clear-cut separation of *L. minuta* from these three species is impossible; however, it would appear to be easily distinguishable from them, as well as from all other species, by the shape of its vegetative cells and by the larger, multinucleate forms which it produces in older cultures.

Table 1. *Reported dimensions of vegetative cells in species of* Labyrinthula

	Species	Observer	Length (μ.)	Width (μ.)
(1)	*L. minuta* sp.nov.	—	5–10	3–5
(2)	*L. macrocystis* Cienkowski	Cienkowski (1867)	18–25	4–5
(3)	*L. macrocystis* Cienkowski	Renn (1936)	10–20	5–8
(4)	*L. macrocystis* Cienkowski	Young (1943)	8·5–30	4
(5)	*L. vitellina* Cienkowski	Cienkowski (1867)	12–17	3–4
(6)	*L. zopfii* Dangeard	Dangeard (1910)	Not given	
(7)	*L. zopfii* Valkanov*	Valkanov (1929)	8	
(8)	*L. cienkowksii* Zopf	Zopf (1892)	11–20	
(9)	*L. chattonii* Dangeard	Dangeard (1932)	Not given	
(10)	*Labyrinthomyxa sauvageaui* Duboscq	Duboscq (1921)	6–8	
(11)	*Pseudoplasmodium aurantiacum* Molisch	Molisch (1926)	7–9	

* Karling changed this species name to *L. valkanovii* (*Amer. J. Bot.* 1944, 31, 50).

Cell shape

All species listed in Table 1 have fusiform vegetative cells (Pl. 1, fig. 3) except *Labyrinthula minuta* which has predominantly oval cells (Pl. 1, figs. 4, 7). Occasionally, they may appear elongated with tapered ends, this aspect appearing when an individual cell attempts to conform to the shape of the slime track. The cells of *L. minuta* exhibit less flexibility than do those of the larger species which frequently bend or constrict when passing a barrier (Pl. 1, fig. 6) or may alternatively become extremely elongated and distorted as they conform to the contour of the track.

Cell motility

Labyrinthula minuta, like other members of this genus, has a gliding type of motility restricted to its preformed slime tracks. It differs from other species of *Labyrinthula* in its much slower rate of movement and in the ability to reverse its direction of movement. The movement of *L. minuta* is extremely slow, 4–5μ./min., and it is perceptible only with a 4 mm. or oil-immersion objective. The rate of movement of only two other species has been reported in the literature; Cienkowski stated that *L. vitellina* moved 12·5–25μ./min. while Young observed *L. macrocystis* to travel at 150μ./min. Considerable variation in rates of movement have been observed in these larger species when cultivated in this laboratory, and oftentimes cells in one area of a given culture would be moving as slowly as 15–30μ./min., whereas those in other areas were moving at the rate indicated by Young. The slow rate of cell movement in *L. minuta* provides a distinctive and valuable differential characteristic for the diagnosis of this species.

Labyrinthula minuta is apparently unique in the capacity of its cells to reverse their direction of movement on a slime track. Of the reports cited in Table 1, only that of Cienkowski provided information on this point, and he was specific in stating that the cells of *L. vitellina* and *L. macrocystis* did not reverse their direction of movement. Such cells never continue movement for more than 20μ. in a given direction. After moving for this or a lesser distance the cells either stop for an indefinite period or immediately reverse their direction. Such a lack of continuous movement is rarely seen in the larger species where cells and groups of cells commonly move one or more millimetres without stopping. Since the movement of *L. minuta* is so slow and is reversed after such short distances, one never observes the mass streaming motility which is so typical of the larger species, and which has come to be accepted as such a distinctive characteristic of the genus *Labyrinthula* (Pl. 2, fig. 8). As one would anticipate, cell divisions may be witnessed most commonly when cells of *L. minuta* remain motionless on the slime track.

Cell division and generation time

The mode of division of *Labyrinthula minuta* deviates markedly from the common transverse fission described for and observed in other species of this genus. Two rapid divisions at right angles to one another, followed by a period of longer duration when the cells increase in size, characterize and distinguish the vegetative multiplication of this species. The first division is transverse and occurs when the cell has reached its maximum dimensions (i.e. 5μ. wide × 10μ. long). The two cells remain attached and often the line of division becomes obscured. The daughter cells usually divide within 30 min. the plane of this second division occurring at right angles to that of the first (Fig. 1).

Following the second division, the cells may move apart immediately, but more commonly they remain in a tetrad formation for several hours prior to separation (Pl. 1, fig. 5). After the second division, each cell increases in size during the next 6–8 hr., by when it has once again reached its maximal size for two successive divisions. Thus, under optimal conditions, four daughter cells may be produced from each single cell on an average of about once every 8 hr. This was determined by observations of growing vegetative cultures, and by isolating single cells and following their increase in number for 32–43 hr. periods. The data for this latter experiment is presented in Table 2.

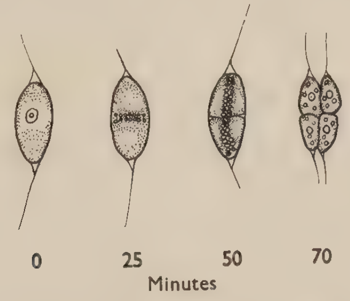


Fig. 1. A cell of *Labyrinthula minuta* dividing into tetrads. Camera lucida drawing. × 1050.

Table 2. *Increase in cell numbers with time from isolated single cells of* Labyrinthula minuta

Time of observation (hr.)	Isolate of *L. minuta*			
	No. 1	No. 2	No. 3	No. 4
	Progeny (no. of cells)			
0	1	1	1	1
5	1	4	4	4
8	4	4	4	4
18	16	16	28	42
19	16	22	60	60
23	44	29	64	68
26	54	54	64	98
32	145	68	126	220*
43	—	—	—	576*

* Estimated.

Nucleus

Because of the peculiar sequence of cell division in *Labyrinthula minuta* just described, nuclear divisions were followed in relationship to cytokinesis. Such divisions could not be followed in living cultures since the cytoplasm obscured the nuclei during this process; however, it was possible to follow this sequence by studying impression smears prepared by the Feulgen reaction or by staining with iron haematoxylin. Mitotic figures were easily seen in these preparations. Before the first cytoplasmic division, the nucleus divides mitotically with the mitotic figure arranged at right angles to the long axis of the cell, and two interkinetic nuclei are seen just before the first cytoplasmic division. Following the primary division, the nuclei again divide mitotically, the mitotic figures in this case being in a plane at an angle of 90° to the first nuclear division. Since two divisions occur in a single cell within an hour the mitotic divisions obviously occur very rapidly, and it is estimated that only 15–20 min. elapse during each nuclear division. During the second division mitosis is synchronous, or nearly so, in the two sister cells.

The interkinetic nucleus observed with the phase-contrast microscope is spherical, 1·5–2 μ. in diameter and is centrally located in the cell (Pl. 1, figs. 4, 7). The nucleolus is 1 μ. in diameter, occupies a central position in the nucleus, stains intensely with iron or Harris's haematoxylin, and is Feulgen negative.

Cytoplasm

The only cytoplasmic structures visible in living cells of *Labyrinthula minuta* are 4–15 small granules 0·2–0·6 μ. in diameter (Pl. 1, fig. 7), distributed at random throughout the cytoplasm, and these become aligned along the axis of division just before the completion of cytokinesis (Fig. 1). Because of this interesting feature the nature of these granules was investigated. In Helly's fixed smears, stained with acid aniline fuchsin and methyl green, one to five mitochondria/cell were demonstrable, these being the size of the smallest granules observed in the living cells. However, no alignment of the mitochondria along the axis of division was seen. When preparations were stained with Sudan Black many lipid granules became apparent which showed the same distribution and alignment as granules seen in living cells during and after division. It seems probable, therefore, that the majority of the granules observed in living cells are lipid storage granules. The larger species also have many granules which are visible in the living cell, disappear when treated with fat solvents, and stain with Sudan Black and other fat stains. Unlike the fat granules in *L. minuta*, those of the larger species do not change position during cytokinesis.

Multinucleate bodies

Labyrinthula minuta is the only member of the genus known to regularly develop relatively large multinucleate structures when grown on a solid agar medium. Such forms occur only within the first centimetre of growth at the periphery of the colony in cultures aged 1 week or more.

These multinucleate bodies vary in size, shape and number of nuclei. In 7-day culture the largest form found was only 10 μ. in diameter (Pl. 2, fig. 13), whereas in 20 to 30-day cultures many bodies were 18–30 μ. in diameter (Pl. 2, figs. 10–12 and 14). These structures are usually oval to round (Pl. 2, fig. 10), but may be oblong or angular when compressed by other cells. They rarely contain more than 4–8 nuclei (Pl. 2, fig. 13) in a 7-day culture, while in month-old cultures they not uncommonly contain 16 to 64 nuclei/body (Pl. 2, figs. 11, 14).

This multinucleate body apparently develops from a single, normal negative cell, although this could not be substantiated by observations of single cells in living culture alone. From the study of stained slides in conjunction with living cultures, it seems probable that the multinucleate body develops by the enlargement of a vegetative cell which undergoes repeated synchronous nuclear divisions in the absence of cytokinesis which is suppressed longer in older cultures than in cultures 1–2 weeks old. In the younger cultures cytoplasmic cleavage is initiated in cells with only 4–8 nuclei, while in older cultures cytokinesis often does not occur until there are *c.* 64 nuclei in one body.

Apparently, the multinucleate bodies cleave eventually into uninucleate vegetative cells (Pl. 2, fig. 12), for only the latter can be observed in the older portions of the expanding colony. However, the complete cleavage of any large body into an appropriate number of uninucleate components has not been observed in the living state. We know that this occurs slowly and is not synchronous. Living multinucleate bodies have been observed continuously for 48 hr., and during this period unequal cleavage of the cytoplasm took place, but the large bodies did not fragment completely into uninucleate cells. In one instance, several uninucleate cells did separate off, but a considerable portion of the large body retained its multinucleate structure. In other instances unequal cleavage resulted in two or more smaller multinucleate bodies and one or two cells with single nuclei. Examination of stained smears led us to conclude that these smaller multinucleate cells eventually cleave into uninucleate cells.

DISCUSSION

If we accept the broad definition of a plasmodium as an unwalled, multinucleate mass of protoplasm, then the term plasmodia can be applied to the multinucleate bodies of *Labyrinthula minuta*. Plasmodia are also found in the Myxomycetes, the Plasmodiophorales, and the Myxosporidiae. The basic common characteristic of the plasmodia of these four groups of organisms is the condition of multinucleation. The four types of plasmodia may be compared and contrasted as to their formation and their resolution.

Since the plasmodium of *Labyrinthula minuta* arises, apparently, from a single non-amoeboid, haploid cell it is unlike the diploid plasmodium of the Myxomycetes and the Plasmodiophorales. These plasmodia arise from the fusion of two swarm cells or myxamoebae. On the other hand, certain of the Plasmodiophorales do possess a second plasmodial stage which arises from a single haploid amoeboid cell. In certain species of the Myxosporidiae haploid amoebulae penetrate host tissue, grow in size, and undergo schizogony cutting off uninucleate schizonts. Each schizont develops into a multinucleate amoeboid plasmodium.

Little or no similarity exists in the ultimate transformation of the mature plasmodium in *Labyrinthula minuta*, the Myxomycetes, the Plasmodiophorales and the Myxosporidiae. In the Myxomycetes the entire plasmodium is converted into one or more fruiting bodies while in the Plasmodiophorales and the Myxosporidiae the plasmodium eventually cleaves into spores. In contrast, the plasmodium of *L. minuta* fragments into uninucleate or multinucleate vegetative cells. Spores have never been seen in this species. Thus, possession of a multinucleate stage is not adequate evidence for relating *L. minuta* to these three other groups of organisms.

In a paper published in 1953, relating to the steroid requirements of two isolates of *Labyrinthula*, Vishniac & Watson applied the name *L. minuta* var. *atlantica* to the organism here described. At that time these investigators believed they had two isolates of a minute and distinctive species, and hence used the above name to distinguish one of these in the laboratory. Unfortunately, the name was carried over into the publication just cited. However, no descriptive information or illustrations whatsoever were published concerning the colonial characteristics of the organism or the morphology and dimensions of its cellular elements. Furthermore, additional study of the two strains has shown them to be identical. The foregoing technical description is presented to validate the name *L. minuta* sp.nov. and the epithet incorrectly used earlier should be abandoned.

Type culture material, consisting of stained slide preparations and photomicrographs, has been deposited in the Farlow Herbarium, Harvard University, Cambridge, Mass., and in the Mycological Herbarium, New York Botanical Garden, Bronx Park, New York, New York.

This investigation was supported in part by a research grant, C-2119M and G, from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

REFERENCES

ARCHER, W. (1875). On *Chlamydomyxa labyrinthuloides*. *Quart. J. micro. Sci.* **15**, N.S., 107.

CIENKOWSKI, L. (1867). Über den Bau und die Entwickelung der *Labyrinthuleen*. *Arch. mikr. Anat.* **3**, 274.

DANGEARD, P. A. (1910). Études sur le développement et la structure des organismes inferieurs. *Botaniste*, **1**, 58.

DANGEARD, P. A. (1932). Observations sur la famille des Labyrinthulées et sur quelques autres parasites des *Cladophora*. *Botaniste*, **24**, 217.

DUBOSCQ, O. (1921). *Labyrinthomyxa sauvageaui* protéomyxée parasite de *Laminaria lejolisii* Sauvageau. *C.R. Soc. Biol., Paris*, **84**, 27.

MOLISCH, H. (1926). *Pseudoplasmodium aurantiacum* n.g. et n.sp. eine neue Acrasiee aus Japan. *Sci. Rep. Tôhoku Univ.*, 4th series, no. 1, 119.

RENN, C. E. (1936). The wasting disease of *Zostera marina*. *Biol. Bull., Woods Hole*, **70**, 148.

VALKANOV, A. (1929). Die Natur und die systematische Stellung der Labyrinthuleen. *Arch. Protistenk*, **67**, 110.

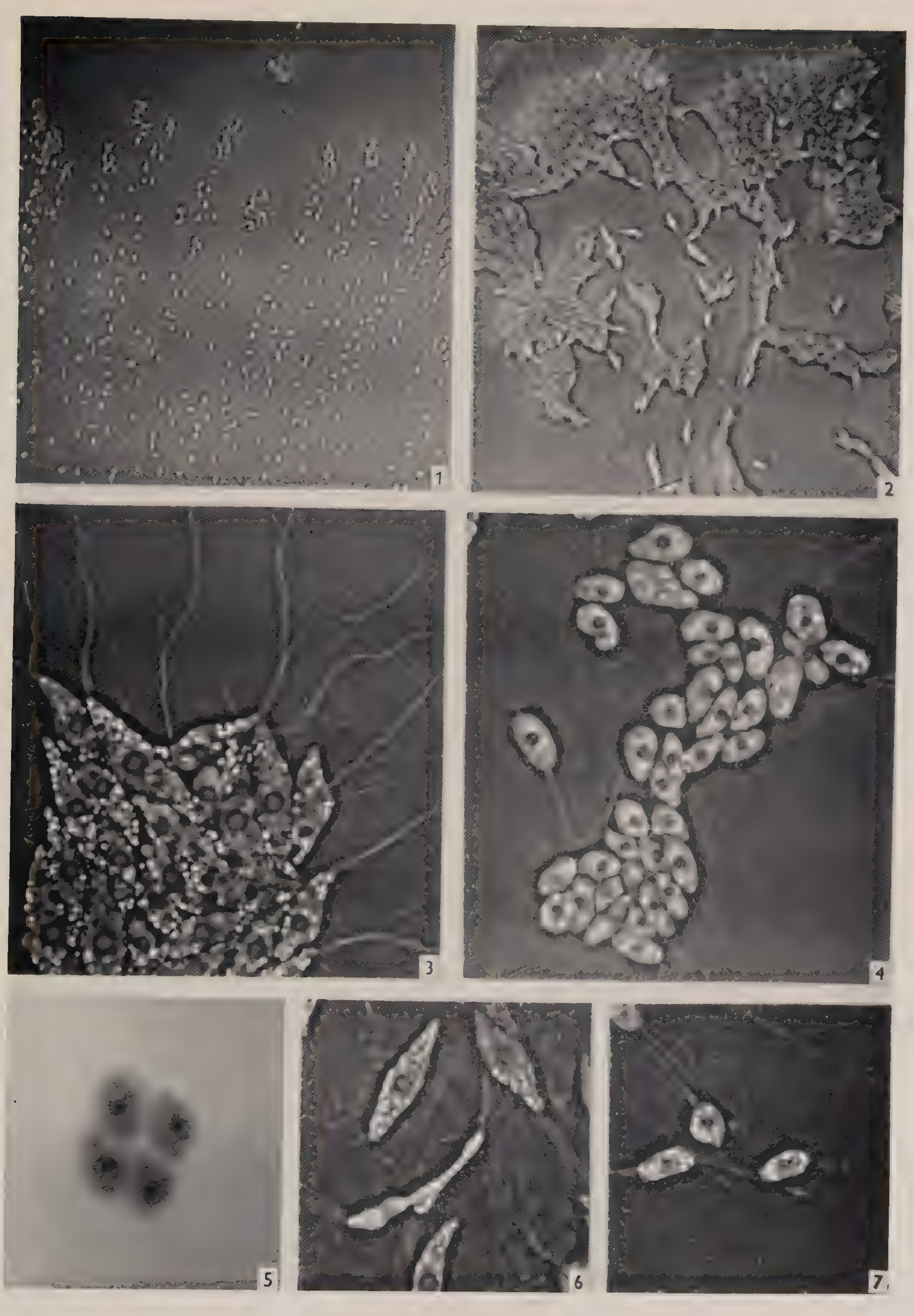
VISCHNIAC, H. S. (1955). The nutritional requirements of isolates of *Labyrinthula* spp *J. gen. Microbiol.* **12**, 455.

VISCHNIAC, H. S. & WATSON, S. W. (1953). The steroid requirements of *Labyrinthula vitellina* var. *pacifica*. *J. gen. Microbiol.* **8**, 248.

WATSON, S. W. & ORDAL, E. J. (1957). Techniques for the isolation of *Labyrinthula* and *Thraustochytrium* in pure culture. *J. Bact.* **73**, 589.

YOUNG, E. L. (1943). Studies on *Labyrinthula* the etiologic agent of the wasting disease of eel-grass. *Amer. J. Bot.* **30**, 586.

ZOPF, W. (1892). Zur Kenntniss der Labyrinthuleen, einer Familie der Mycetozoen. *Beit. Physiol. Morph. nied. Organ.* **2**, 36.



S. W. WATSON AND K. B. RAPER—*LABYRINTHULA MINUTA* SP.NOV. PLATE 1

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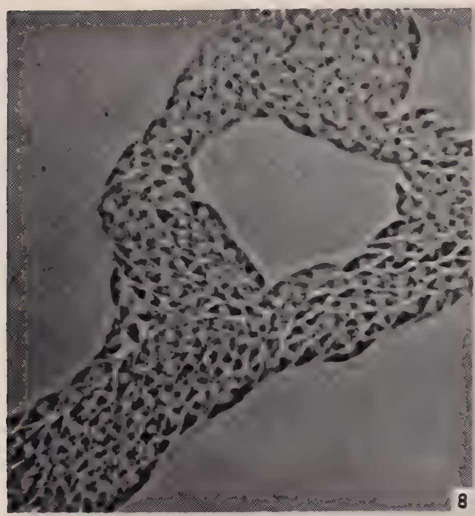
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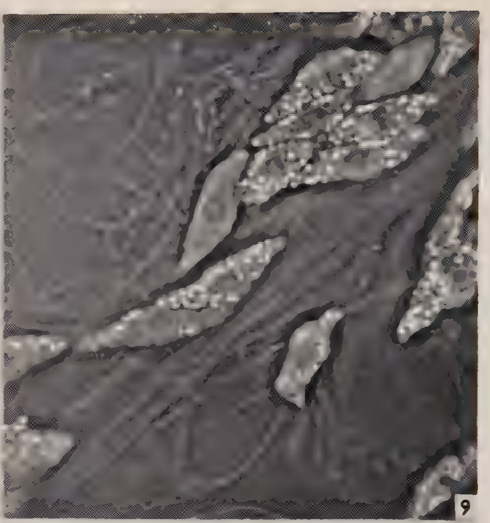
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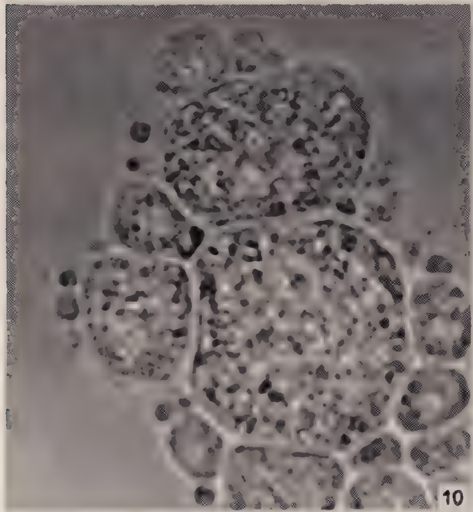
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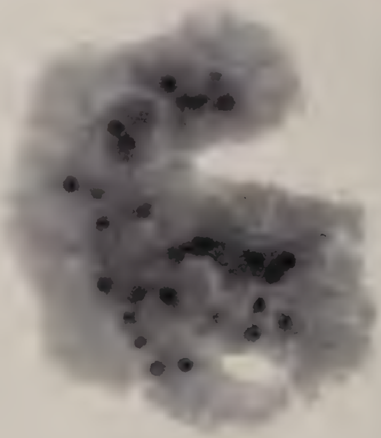
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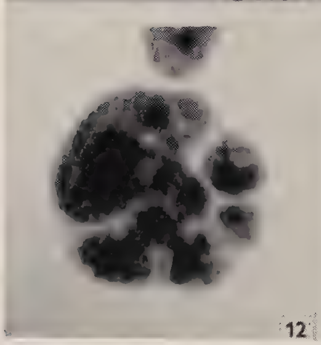
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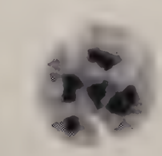
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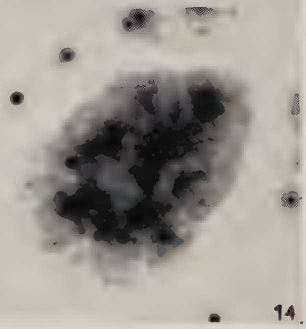
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S. W. Watson and K. B. Raper—*Labyrinthula minuta* sp.nov. Plate 2

EXPLANATION OF PLATES

Plate 1

Fig. 1. *Labyrinthula minuta* growing on an agar surface. Phase-contrast photomicrograph showing distribution of cells in a colony. ×150.

Fig. 2. *L. vitellina* growing on an agar surface showing the net-plasmodium. ×150.

Fig. 3. *L. vitellina* growing on an agar surface. A phase-contrast photomicrograph showing fusiform cells at the edge of a colony. ×900.

Fig. 4. *L. minuta* growing on an agar surface. A phase-contrast photomicrograph showing oval cells with centrally located nuclei. ×900.

Fig. 5. *L. minuta* stained with iron-haematoxylin showing arrangement of cells in a tetrad. ×1425.

Fig. 6. *L. vitellina* growing on an agar surface. Phase-contrast photomicrograph showing distortion of a cell during migration. ×900.

Fig. 7. *L. minuta* growing on an agar surface. Phase-contrast photomicrograph showing shape of cells, internal structures, and the slime filaments connecting the cells. ×900.

Plate 2

Fig. 8. *L. vitellina* growing on an agar surface. Phase-contrast photomicrograph showing many cells moving abreast on portion of the net-plasmodium. ×250.

Fig. 9. *L. vitellina* growing on an agar surface. Phase-contrast photomicrograph showing broad tracks of the net-plasmodium. ×900.

Fig. 10. *L. minuta* growing on an agar surface showing large multinucleate bodies of varied dimensions. ×1425.

Fig. 11. *L. minuta* stained with iron haematoxylin showing multinucleate large body from a 30-day culture. ×1900.

Fig. 12. *L. minuta* stained with iron haematoxylin showing fragmentation of multinucleate body into uninucleate cells. ×1425.

Fig. 13. *L. minuta* stained with iron haematoxylin showing the initiation of cleavage of a small multinucleate body from a 7-day culture. ×1425.

Fig. 14. *L. minuta* stained with iron haematoxylin showing multinucleate body from a 30-day culture. ×1425.

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Littlewood, D. & Postgate, J. R. (1957). *J. gen. Microbiol.* **17**, 378–389

Sodium Chloride and the Growth of *Desulphovibrio desulphuricans*

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SUMMARY: The effect of sodium chloride on the multiplication of four fresh-water and three salt-water strains of sulphate-reducing bacteria was examined by determining the numbers of organisms in a known population which were viable in media of unfamiliar NaCl concentrations. The salt-water strains all produced variants viable at high NaCl concentrations; a population able to multiply in 1 %–10 % NaCl was obtained by 'training' one salt-water strain. They differed, however, in the frequency with which non-exacting variants appeared, ranging from a strain in which the whole population grew with 0·25 % NaCl to one which would not grow with less than 0·45 % NaCl even after repeated attempts at acclimatization. Replacement experiments indicated that the exigent strain required chloride ion; a less-exacting strain required sodium ion. The fresh-water strains produced few variants viable at NaCl concentrations above 3 %, but one strain produced variants of the salt-water type after 'training' to grow with 4 % NaCl; sodium ion was mainly responsible for the inhibition of this strain by high NaCl concentrations. We conclude that the differences among the salt- and fresh-water strains of *Desulphovibrio* do not at present justify their separation into the two species *aestaurii* and *desulphuricans*.

The sulphate-reducing bacteria are common inhabitants of marine, estuarine and fresh-water environments, and laboratory strains frequently show wide differences in tolerance of NaCl. Whether these differences are sufficient to justify a separate classification of marine and fresh-water types has been disputed. Van Delden (1903) tentatively distinguished between *Microspira* (now *Desulphovibrio*) *desulphuricans*, the fresh-water species, and *Microspira aestuarii*, the salt-water species, on the grounds that neither would multiply in a medium containing the salt concentration appropriate to the other. Gahl & Anderson (1928) found that the majority of strains obtained from oil-well water grew with 0–3 % NaCl, but that minorities of exacting or salt-sensitive types existed. They inclined to the view that the exigent and sensitive strains were variants of *desulphuricans*, the prevalent species. Baars (1930) and Kluyver & Baars (1932) did not favour a separate species *aestuarii*, having shown that fresh-water strains could be 'trained' to multiply in a medium containing 3 % NaCl, and that marine strains could undergo the reverse change; but Rittenberg (1941) was unable to repeat the latter observation with two marine strains. Miller (1949) showed that fresh-water strains were readily trained to grow in media containing 4 % NaCl, and grew more rapidly after one passage in such a medium. As a result of tests with many strains originating from sulphur domes he recommended 1 % NaCl as the salt concentration permitting fastest growth and most sulphide formation. Baars (1930) noted that NaCl at 0·6–0·9 % stimulated sulphide production by fresh-water strains, a phenomenon which he believed partly accounted for an increased H_2S production that occurred in Dutch canals when sea water was admitted. Sulphate-reducing bacteria tolerating 5 % NaCl or more are well known: ZoBell & Rittenberg (1948) mentioned several instances of high salt tolerance in their review of the earlier literature; further examples are strain Canet 41 which multiplied in presence of 0–12 % NaCl (Senez, 1951), and Iya & Sreenivasaya's (1945) strain which multiplied in presence of 0–13 % NaCl, most rapid multiplication occurring with 6 % NaCl.

Negative results in growth experiments with sulphate-reducing bacteria can sometimes be attributed to inadequate control of the redox potential of the media, particularly when small inocula are used (Grossman & Postgate, 1953*a*); difficulties of this kind might account for some of the discrepant results reported in the earlier literature. The present paper reports an examination of the salt tolerance of seven strains of sulphate-reducing bacteria (four fresh-water strains and three strains of salt-water origin) in media in which the redox potential was poised with cysteine. In order to judge whether salt tolerance resembled an 'adaptive' or 'mutational' phenomenon, the numbers of organisms of each strain which were viable at different salt concentrations were determined. The nature of the ions concerned in the salt relationships of some of these strains has been studied in greater detail by replacement studies such as have been used with the 'strong' halophiles (review by Flannery, 1956).

METHODS

Organisms. Seven strains called *Desulphovibrio desulphuricans* were used: Beckton 1, a fresh-water strain isolated from sewage sludge (National Collection of Industrial Bacteria [NCIB] 8319); Hildenborough (fresh water) from clay (NCIB 8303); Teddington R (fresh water) from Thames mud (NCIB 8312); Wandle (fresh water) from the River Wandle at Wandsworth (NCIB 8305); British Guiana (of salt-water origin) from 'sling mud' (NCIB 8403); Canet 41 (salt-water origin) from Etang de Canet, Perpignan, France (kindly given by Dr J. Senez; NCIB 8393); El Agheila Z (salt-water origin) from a sulphur-producing lake in Libya (NCIB 8380) (Adams, Butlin, Hollands & Postgate, 1951). The strains British Guiana, El Agheila Z, Hildenborough and Wandle were originally isolated by the method of Butlin, Adams & Thomas (1949) using $Na_2SO_3.7H_2O$ (3 %) to hinder the growth of contaminants; Beckton 1, Canet 41 and Teddington R were isolated without this.

Cultivation. Stocks were kept in a freeze-dried condition, but during work on a particular strain it was subcultured weekly in the lactate + yeast extract + sulphate medium C of Butlin *et al.* (1949) made up with analytical grade reagents where available. This medium contained no added NaCl, but small amounts of sodium and chloride ions were present (0·014 % (w/v) Na^+, 0·065 % (w/v) Cl^-; all percentages quoted in this paper are w/v except where otherwise stated). Cultures (usually 5 ml. in $6 \times \frac{5}{8}$ in. test tubes) were incubated at 30° in McIntosh & Fildes anaerobic jars containing $H_2 + CO_2$ (1 %, v/v). For experiments requiring large quantities of organisms, medium C was supplemented with tryptone (Oxoid, 0·5 %) and the yeast extract brought up to 0·4 %. Such cultures were grown in 2 l. flasks with a plug containing 1:1 (v/v) pyrogallol (sat.) and Na_2CO_3 (sat.) or in 400 ml. flasks in anerobic jars; the bacteria were harvested by centrifugation after 4 days of incubation. All media were supplemented with cysteine hydrochloride (5 μmole/ml.) and a trace of ferrous ammonium sulphate (4 μg./ml.). Media for natural or induced salt-tolerant strains were supplemented with the requisite amounts of NaCl. All media and solutions to be added thereto were sterilized by autoclaving except the cysteine hydrochloride solution which was sterilized by Seitz filtration. The pH value was finally adjusted to 7·4 ± 0·2 with sterile N-NaOH.

Counting procedures. Viable counts were carried out by the method of Grossman & Postgate (1953*b*). Serial decimal dilutions (0·5 ml. → 5 ml.) of a fully grown culture were made in a mineral solution consisting of medium C without lactate, yeast extract or sulphate (supplemented with 2·5 % NaCl for salt-water strains) and 0·5 ml. samples of the dilutions were tested in quintuplicate in 4·5 ml. portions of medium C supplemented with the appropriate quantity of NaCl, cysteine hydrochloride and sufficient ferrous ammonium sulphate to indicate growth by blackening (0·05 %). In certain instances $MgCl_2$ or Na_2SO_4 replaced some or all of the NaCl in the test medium. By this procedure the test media contained, when finally inoculated, extra NaCl equivalent to one-in-ten dilution of the mineral solution in which the culture being tested was diluted. With fresh-water strains this error was ignored; with salt-water strains carry-over amounted to 0·25 % NaCl, and allowance was made for this in preparing the test media. Because of this carry-over, 0·25 % NaCl was the lowest salt concentration in which salt-water organisms were usually tested. The tubes were incubated until no further blackening was observed. The most probable numbers (M.P.N) of viable bacteria in the original sample were then obtained from statistical tables (Min. of Health, 1939); counts recorded in the figures as log M.P.N./ml. = −0·6 represent experiments in which no growth occurred after quintuplicate decimal (0·5 ml. → 5 ml.) dilution of the parent culture directly into the test medium; they thus signify an absolute M.P.N. of less than 2 viable organisms/5 ml. culture, the usual volume of the cultures examined during this work. In certain tests with strain British Guiana the procedure was scaled up in an attempt to detect viable salt-tolerant variants of even lower M.P.N./ml.; the decimal dilutions from the parent culture were 5 ml. → 50 ml. as well as the higher dilutions mentioned already.

Total counts were determined by diluting a culture in the appropriate mineral solution, heating to abolish motility and counting microscopically in a Thoma haemocytometer (Hawksley Ltd., London) of 0·02 mm. depth.

Lysis experiments. Organisms of ‘strong’ halophiles sometimes burst on transfer to distilled water, owing to entry of water by osmosis (Christian, 1956). Attempts to demonstrate osmotic bursting with certain salt-tolerant strains of *Desulphovibrio desulphuricans* were made by adding small volumes of a thick suspension of organisms to saline and to distilled water and comparing the turbidities of the suspensions at intervals. The procedure was checked by using the halophilic organism NCIB 8668 (kindly given by Professor F. Egami) which showed an immediate decrease in turbidity in distilled water to give a final optical density 16 % the original value in 1 min.

Optical density measurements. These were carried out in the E.E.L. colorimeter (Evans Electroselenium Ltd., Harlow, Essex) calibrated with the Hildenborough strain of *Desulphovibrio desulphuricans.*

Irradiation with ultraviolet light. Samples from a fully grown culture were irradiated from a 7 W. jacketed 12 in. Hanovia UV lamp (Hanovia Ltd., Slough) emitting maximally at 253·7 μ. The culture fluid was 2 mm. deep, and treated in a Petri dish 35 cm. from the radiation source.

RESULTS

Distribution of NaCl-*tolerance in 'normal' populations*

The numbers of bacteria viable in media of different NaCl concentrations were determined using fully grown cultures of the 7 strains of *Desulphovibrio desulphuricans*. The distribution curves obtained (Figs. 1, 2) can be divided into two main types.

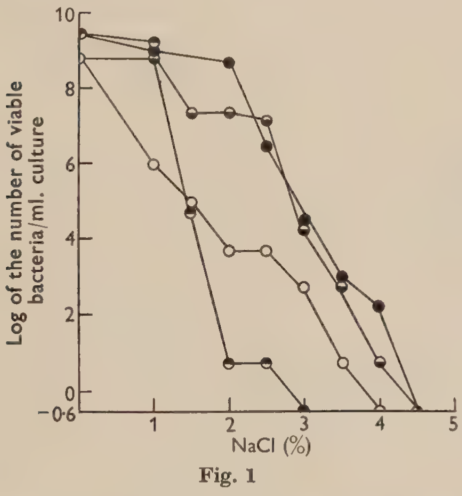

Fig. 1

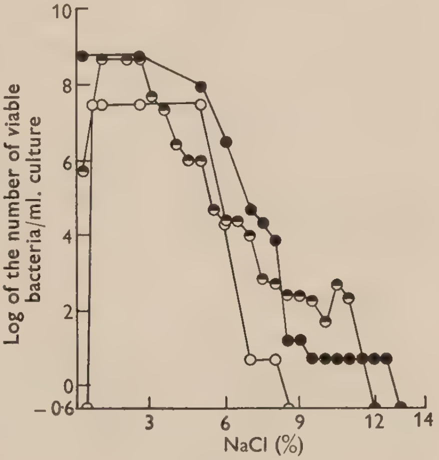

Fig. 2

Fig. 1. Numbers of four fresh-water strains of *Desulphovibrio desulphuricans* viable in media of various NaCl concentrations. ●, Hildenborough; ◒, Wandle; ◓, Teddington R; ○, Beckton 1.

Fig. 2. Numbers of three salt water strains of *Desulphovibrio desulphuricans* viable in media of various NaCl concentrations. ○, British Guiana; ●, Canet 41; ◓, El Agheila Z.

Fresh-water organisms

Strains Beckton 1, Teddington R, Hildenborough and Wandle, normally grown in medium C without extra NaCl, showed a relatively small range of salt tolerance; salt-tolerant variants did not appear above 3–4 % NaCl. Strains Hildenborough and Wandle showed a wider range of salt tolerance than the other two strains, possibly because of the procedure used during their isolation (see Discussion). Strain Hildenborough showed atypical growth in higher salt concentration (3–4 % NaCl); the bacteria were associated with the precipitate and blackening did not extend throughout the supernatant medium.

Salt-water organisms

The three organisms isolated from saline environments were normally grown in medium C + 2·5 % extra NaCl. They differed markedly from the fresh-water types in that variants tolerating high NaCl concentrations were present in normal populations. These three organisms differed on the basis of the population distributions in subnormal NaCl concentrations.

Strain British Guiana. This strain showed true halophily in the sense that none of the bacteria was viable with a low (0·4 %) NaCl concentration. When a large innoculum was used (0·5 ml. or 5 ml. parent culture) a black precipitate of ferrous sulphide often developed in the subculture, but when this was examined microscopically no bacteria could be seen adhering to the particles, and none was removed by washing the precipitate. A maximum viable count was obtained in 0·6 % NaCl, but in 0·5 % NaCl the results varied considerably from experiment to experiment and were irregular in that tubes from higher dilutions sometimes gave blackening when tubes from lower dilutions did not.

Strain Canet 41. The whole population of this strain was viable in 0·25 % NaCl. This strain showed the widest range of salt tolerance, confirming and extending the original observation of Senez that cultures of Canet 41 grew with 0–12 % NaCl (Senez, 1951).

Strain El Agheila Z. This strain showed an intermediate behaviour in which most of the organisms were not viable with 0·25 % NaCl, but some non-exacting variants were present; the proportion of the latter was higher in young cultures (Table 1) than in older ones.

Table 1. *Effect of age of parent culture on the viability of* Desulphovibrio desulphuricans *strain El Agheila Z in media of low salt concentration*

The viable populations in cultures of different ages were counted in media containing: (*a*) 1 % NaCl (in which a maximum viable count was obtained); (*b*) 0·25 % NaCl; (*c*) 0·25 % NaCl + $MgCl_2$ to Cl' concentration equivalent to (*a*). The figures quoted are numbers of viable bacteria/ml. original culture.

Age of parent culture (days)	1 % NaCl (0·6 % Cl')	0·25% NaCl	0·25 % NaCl + 0·61 % $MgCl_2$ (total $[Cl'] = 0·6$ %)
3	5×10^8	$3·4 \times 10^6$	1×10^8
6	5×10^8	5×10^5	7×10^7
7	5×10^8	$1·4 \times 10^4$	5×10^6

Distribution of NaCl *tolerance in 'trained' populations*

Strain Wandle was 'trained' to tolerate NaCl by subculturing eleven times in medium C containing 4 % NaCl. The distribution of salt-tolerant variants in a population grown in this medium was then comparable with that of Canet 41, the most salt-tolerant organism examined (Fig. 3).

Strain Canet 41 was trained to high salt resistance by twelve subcultures in medium C containing 10 % NaCl. The whole population of such a culture was then viable in NaCl concentrations ranging from 1 to 10 % and variants tolerating up to 16 % NaCl were present (Fig. 4).

Three separate attempts to train strain British Guiana to grow with 0·4% NaCl were not successful. Cultures able to grow with 0·5 % or 0·45 % NaCl were obtained without much difficulty, but even after several subcultures at these salt concentrations the trained cultures would not grow with 0·4 % or less NaCl. The substrain trained to grow with 0·45 % NaCl was irradiated with u.v.-radiation for 40 sec., which gave about 1 % survival of the parent strain, but no variants able to grow with 0·3 % or less NaCl were obtained.

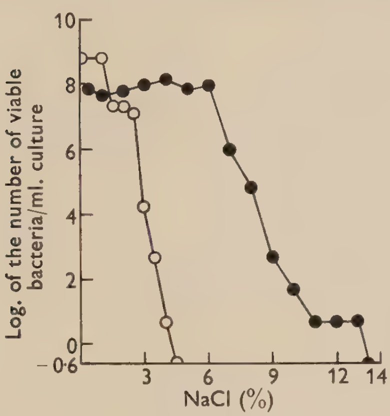

Fig. 3

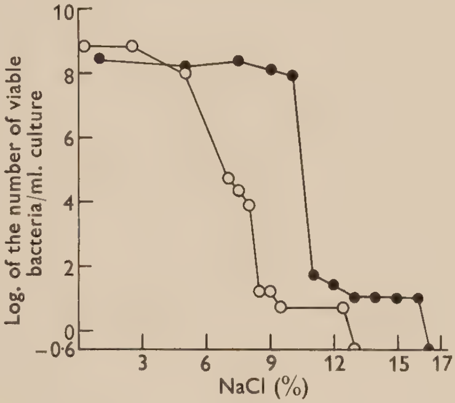

Fig. 4

Fig. 3. Numbers of ***Desulphovibrio desulphuricans*** strain Wandle and of a sub-strain trained to 4% NaCl viable in media of various NaCl concentrations. ○, normal strain; ●, adapted strain.

Fig. 4. Numbers of ***Desulphovibrio desulphuricans*** strain Canet 41 and of a sub-strain trained to 10 % NaCl viable in media of various NaCl concentrations. ○, normal strain; ●, adapted strain.

During training the cultures were morphologically unusual and multiplication tended to be restricted to the precipitate in the medium. Few typical vibros were visible under the microscope and many sluggishly motile fat spirilloid organisms, some 50μ. long, were seen. Morphologically aberrant forms in unfamiliar salt concentration have been reported with sulphate-reducing bacteria (Baars, 1930; Rittenberg, 1941) as with other bacteria (Flannery, 1956). The substrain acclimatized to 0·45 % NaCl became morphologically more normal, however, after five or more subcultures in this salt concentration and typical vibrios then appeared throughout the medium, though a few spirilloid forms persisted.

Ions responsible for the effects of NaCl *on growth*

Flannery, Doetsch & Hansen (1952) showed by replacement experiments with other salts that the 'strong' halophile *Vibrio costicolus* required sodium rather than chloride ion for multiplication; Robinson & Gibbons (1952) observed that *Micrococcus denitrificans* required sodium chloride specifically for multiplication. Replacement experiments were used to study the nature of the effects of NaCl on the growth of various strains of *Desulphovibrio desulphuricans*.

Strain Wandle. This strain was tested in media in which Na_2SO_4 or $MgCl_2$ replaced NaCl (Fig. 5). The distribution of salt-tolerant variants in Na_2SO_4 was similar to that in NaCl provided the concentrations were expressed in % Na^+; the organisms tolerated a much higher concentration of Cl′ when the cation was Mg^{++}. Hence, subject to certain provisos about the dissociation of these salts (see Discussion), the inhibitory effect of NaCl is attributable to the sodium ion; it is neither a non-specific osmotic effect nor due to chloride ion.

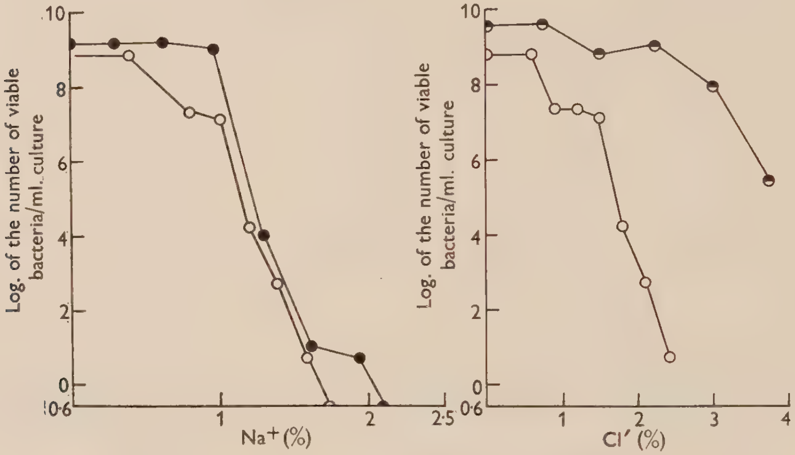

Fig. 5. Numbers of *Desulphovibrio desulphuricans* strain Wandle viable in media containing various concentrations of NaCl, Na_2SO_4 or $MgCl_2$. ○, NaCl; ●, Na_2SO_4; ◓, $MgCl_2$.

Strain El Agheila Z. The requirement of the majority of organisms in a 'normal' population of strain El Agheila Z was shown by a similar procedure to be for sodium rather than for chloride ion (Table 1). Na_2SO_4, added to the medium so that the final concentration of Na^+ was equivalent to 1 % NaCl, permitted the whole population to multiply; $MgCl_2$, added to give an equivalent Cl′ concentration, did not do this. $MgCl_2$ had a partial synergistic effect with NaCl (Table 1) which was most pronounced with young cultures; it was not studied further.

The inhibitory effect of high concentrations of NaCl on strain El Agheila Z could not be attributed solely to sodium ion. A 'normal' culture contained about 26 organisms/ml. which were viable in 9 % NaCl, but when media were made up with 1 % NaCl $+Na_2SO_4$ together equivalent in Na^+ to 9 % NaCl, or with 1 % $NaCl+MgCl_2$ together equivalent in Cl′ to 9 % NaCl, no viable bacteria were detected.

Strain British Guiana. The requirement of this strain for NaCl was examined in a similar manner to that used with strain El Agheila Z. A maximum viable count was obtained with 1 % NaCl or with $MgCl_2$ added to the medium so that the final Cl′ concentration was equivalent to 1 % NaCl. With Na_2SO_4 added to give a final Na^+ concentration equivalent to 1 % NaCl, however, a maximum count was not obtained; the results of tests with Na_2SO_4 were very irregular and recalled the dilution counts in 0·5 % NaCl referred to earlier. Hence, in contrast to strain El Agheila Z, strain British Guiana required chloride ion rather than sodium ion for multiplication.

The inhibition of strain British Guiana by high salt concentrations could not be attributed solely either to Na^+ or Cl′; $2{\cdot}2 \times 10^4$ organisms/ml. were viable with 6 % NaCl but only 7×10^2/ml. grew in a medium containing 1 % NaCl + Na_2SO_4 together equivalent in Na^+ to 6 % NaCl, and only 5×10^2/ml. grew in a medium containing 1 % NaCl + $MgCl_2$ together equivalent in Cl′ to 6 % NaCl. As with strain El Agheila Z, the mixed salts were more inhibitory than NaCl alone.

Attempts to observe osmotic lysis. No lysis was detected turbidimetrically when strain British Guiana, strain Wandle trained to multiply in 4 % NaCl or strain Canet 41 trained to multiply in 10 % NaCl were exposed to distilled water (0·27–0·42 mg. dry wt. bacteria/ml.) for 2½–3 hr. at room temperature.

Ratio of viable to total count. The procedure used in this work for estimating viable bacteria was that of Grossman & Postgate (1953*b*). These authors obtained viable counts amounting to between 20 and 70 % of the total count, the error being mainly attributable to losses during manipulation. The percentage recoveries of viable bacteria obtained in the present work were of similar orders (Table 2) with the exception of strain British Guiana, suggesting that high manipulative losses occurred with this strain.

Table 2. *Relationship between viable counts and total counts of seven strains of* Desulphovibrio desulphuricans

Strain	Viable organisms in original culture (no./ml.)	Total number of organisms visible in haemocytometer (no./ml.)	Viability (% total count)
Beckton 1	$1{\cdot}0 \times 10^9$	$1{\cdot}4 \times 10^9$	71·4
Hildenborough	$1{\cdot}0 \times 10^9$	$1{\cdot}4 \times 10^9$	71·4
Teddington R	$1{\cdot}0 \times 10^9$	$1{\cdot}6 \times 10^9$	62·5
Wandle	$7{\cdot}0 \times 10^8$	$1{\cdot}14 \times 10^9$	61·4
British Guiana	$3{\cdot}4 \times 10^7$	$4{\cdot}4 \times 10^8$	7·7
Canet 41	$7{\cdot}0 \times 10^8$	$1{\cdot}3 \times 10^9$	53·8
El Agheila Z	$2{\cdot}6 \times 10^8$	$9{\cdot}25 \times 10^8$	28·1

DISCUSSION

Nature of the effect of NaCl *on growth.* The experiments in which NaCl was replaced by Na_2SO_4 or $MgCl_2$ indicated that the requirement of strain El Agheila Z was primarily for sodium ion, and that this ion was also primarily responsible for the inhibitory effects of NaCl on the viability of strain Wandle. These strains thus resemble *Vibro costicolus* (Flannery, 1952) in that sodium ion is of primary importance in their growth. On the other hand, the requirement of strain British Guiana was primarily for chloride ion and not sodium ion; thus it resembled neither *V. costicolus* nor *Micrococcus denitrificans* (Robinson & Gibbons, 1952).

The significance of replacement experiments on inhibition of multiplication by strong NaCl solutions can be criticized on the grounds that, if $MgCl_2$ were imperfectly dissociated like $CdCl_2$ (strong solutions of which contain much of the ionic species $CdCl^+$ and hence have a chloride ion concentration lower than theoretical), then the actual chloride ion concentration in the tests reported here might be much lower than the total added chloride would indicate. This possibility was examined by plotting the curves relating the equivalent conductivity to the concentration of NaCl and $MgCl_2$ derived from International Critical Tables and interpolating the values giving comparable recoveries of viable organisms of strain Wandle. Even if the whole of the deviation from theoretical of $MgCl_2$ were attributed to an association of the type $Mg^{++} + Cl' \rightarrow MgCl^+$, the chloride ion concentration would still have been *c.* 35 % greater than in the equivalent NaCl solution. The effect of sodium ion on the growth of strains Wandle and El Agheila Z emphasizes the fact, mentioned by Butlin *et al.* (1949), that 3 % $Na_2SO_3.7H_2O$ should not be used to suppress contaminants if obligate non-halophilic strains of *Desulphovibrio desulphuricans* are being isolated, because a 3 % solution of $Na_2SO_3.7H_2O$ has a Na^+ concentration equivalent to 1·4 % NaCl. Strains Hildenborough and Wandle were isolated by this procedure and this may account for their higher salt tolerance compared with strains Teddington R and Beckton 1; strain El Agheila Z was also treated with 3 % $Na_2SO_3.7H_2O$ (thus passing through a medium of Na^+ concentration equivalent to 3·9 % NaCl) which may have accentuated its tendency to halophily. All media used in the present work, even when not supplemented with NaCl, contained low concentrations of Na^+ and Cl'; for this reason experiments similar to those of Ware, Childs & Smith (1955) on faecal organisms, to determine the effects of very low concentrations of NaCl on growth, were not carried out.

Most of the strains examined in this work, whether of salt- or fresh-water origin, gave maximum viabilities in 1 % NaCl, supporting Miller's (1949) contention that 1 % NaCl is most satisfactory for growth of both types of sulphate-reducing bacteria. However, strain Beckton 1 was an exception and only a small proportion of the population was viable in 1 % NaCl. In spite of Gahl & Anderson's (1928) demonstration of salt-sensitive strains in oil-well waters, media supplemented with 1 % NaCl have been recommended as 'general purpose' media for the growth of sulphate-reducing bacteria. The existence of very salt-sensitive strains such as strain Beckton 1 confirms that such media should be used cautiously.

Halophily and nomenclature in Desulphovibrio. The distribution patterns of salt tolerance in the strains studied here ranged from the strict NaCl requirements of strain British Guiana, through the partial NaCl requirement of strain El Agheila Z and the wide salt tolerance of strain Canet 41, to the comparative salt-sensitivity of the fresh-water strains. Thus there seems to be little difference of kind between the salt-water and fresh-water types: they differ mainly in the number of variants produced which are able to tolerate particularly high or low salt concentrations. Whether these variants should properly be called mutants depends more on one's definition of the term mutant than on the experimental data presented here, for there is no obvious qualitative difference between the few variants of strain Canet 41 which are able to grow in 10 % NaCl (*c.* 1 organism in 10^8), which one would normally call mutants and the many able to grow with 6 % NaCl (*c.* 1 organism in 100) which are far more plentiful than classical mutants. The procedure used in this work would not have detected variants if their proportion was below *c.* 1 in 10^9 ordinary organisms, and it may well be that strain British Guiana is an extreme form of the El Agheila Z type, capable in principle of producing non-exacting variants, but at so low a frequency that the methods used here did not detect them. These strains differed in the character of their sodium chloride requirements, one requiring primarily sodium ion and the other requiring primarily chloride ion, and it is not unreasonable that the frequency with which 'chloride-less' variants arise should differ from the frequency with which 'sodium-less' variants occur.

For similar reasons the question which of these strains should properly be termed 'halophilic' has, like the mutant question, a semantic flavour. None of them, not even strain Canet 41 after training to multiply in 10 % NaCl, showed the osmotic lysis in distilled water characteristic of the more exacting halophiles of other genera studied by Christian (1956). Yet because it required some NaCl for growth, strain British Guiana might reasonably be called a halophile, but strain Canet 41 should then be termed a facultative halophile in spite of its marine origin. Strain El Agheila Z is intermediate in behaviour but is still a facultative halophile since a proportion of the population grew without added NaCl.

ZoBell, in *Bergey's Manual* (1948), decided to retain the specific name *aestuarii* for salt-requiring members of the genus *Desulphovibrio* because of Rittenberg's exception to Baars's report that marine strains could be trained to grow without NaCl. In the present work strains Canet 41 and El Agheila Z conformed to Baars's observations, but we were unable to 'train' strain British Guiana to grow without extra NaCl; the behaviour of this strain is thus comparable with the two studied by Rittenberg (1941) and reported by ZoBell & Rittenberg (1948). The apparent divergence between the results of Rittenberg and those of Baars can be reconciled if Rittenberg be regarded as having selected accidentally two strains of British Guiana type. Baars regularly added H_2S water to his cultures at inoculation, but Rittenberg's media did not include a chemical reducing agent, and it is therefore possible that, even had his strains been of the El Agheila Z type (*c.* 0·1 % of the population viable without extra NaCl) he would have failed to 'train' them. In our laboratory, strain El Agheila Z was believed to be an exacting halophile until the present work was undertaken; it now seems clear that the failure of this strain to grow in unsupplemented media, a failure which contrasted with the behaviour of strain Canet 41, was because the customary 1/100 inoculum was effectively $1/10^5$ in terms of organisms viable without added salt. An inoculum of $1/10^5$ is unlikely to grow without added reducing agent (Grossman & Postgate, 1953*a*).

The present work has shown a complete gradation of behaviour towards NaCl within the genus *Desulphovibrio*, ranging from very salt-sensitive types to salt-requiring types; at no point in this gradation is the difference from the neighbouring type large. The question arises whether the use of the specific name *aestuarii* for salt-requiring strains is justified at all. Certainly the name cannot be applied automatically to strains of marine origin since these may be of the Canet 41 or El Agheila Z types, which are clearly facultatively halophilic strains of *D. desulphuricans*, resembling the acclimatized sub-strain of Wandle. It could be argued that the name *D. aestuarii* might be retained, but be restricted to salt-water strains of the British Guiana type. The present authors incline to the opinion that the differences so far established between this strain and the others, i.e. requirement for Cl′ rather than Na^+, and low rate of production of non-exacting variants, are hardly sufficient to justify separate species status unless supported by further morphological and biochemical differences. These are not yet apparent.

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REFERENCES

Adams, M. E., Butlin, K. R., Hollands, S. J. & Postgate, J. R. (1951). The role of hydrogenase in the autotrophy of *Desulphovibrio*. *Research, Lond.* **4**, 245.

Baars, J. K. (1930). *Over sulfaatreductie door bacterien.* Dissertation, Meinema, Delft.

Bergey's Manual of Determinative Bacteriology. Ed. by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. (1948). Baltimore: The Williams & Wilkins Co.

Butlin, K. R., Adams, M. E. & Thomas, M. (1949). The isolation and cultivation of sulphate-reducing bacteria. *J. gen. Microbiol.* **3**, 46.

Christian, J. H. B. (1956). Lysis on dilution of bacteria grown in high salt concentrations. *J. gen. Microbiol.* **15**, vi.

Delden, A. van (1903). Beitrag zur Kenntnis der Sulfatreduktion durch Bakterien. *Zbl. Bakt.* (2. Abt.), **11**, 113.

Flannery, W. L. (1956). Current status of knowledge of halophilic bacteria. *Bact. Rev.* **20**, 49.

Flannery, W. L., Doetsch, R. N. & Hansen, P. A. (1952). Salt desideratum of *Vibrio costicolus*, an obligate halophilic bacterium. 1. Ionic replacement of sodium chloride requirement. *J. Bact.* **64**, 713.

Gahl, R. & Anderson, B. (1928). Sulphate reducing bacteria in California oil waters. *Zbl. Bakt.* (2. Abt.), **73**, 331.

Grossman, J. P. & Postgate, J. R. (1953*a*). Cultivation of sulphate-reducing bacteria. *Nature, Lond.* **171**, 600.

Grossman, J. P. & Postgate, J. R. (1953*b*). The estimation of sulphate-reducing bacteria (*D. desulphuricans*). *Proc. Soc. appl. Bact.* **16**, 1.

Iya, K. K. & Sreenivasaya, M. (1945). Studies in the sulphur formation at Kona, Masulipatam-II. *Curr. Sci.* **14**, 267.

Kluyver, A. J. & Baars, J. K. (1932). On some physiological artefacts. *Proc. Acad. Sci. Amst.* **35**, 370.

Miller, L. P. (1949). Rapid formation of high concentrations of hydrogen sulphide by sulfate-reducing bacteria. *Contr. Boyce Thompson Inst.* **15**, 437.

Ministry of Health (1939). The bacteriological examination of water supplies. *Rep. publ. Hlth med. Subj., Lond.*, no. 71, 2nd ed. London: H.M.S.O.

Rittenberg, S. C. (1941). *Studies on marine sulfate-reducing bacteria.* Dissertation no. 650, Univ. Calif., La Jolla.

Robinson, J. & Gibbons, N. E. (1952). The effect of salts on the growth of *Micrococcus halodenitrificans* (n.sp). *Canad. J. Bot.* **30**, 147.

Senez, J. (1951). Étude comparative de la croissance de *Sporovibrio desulfuricans* sur pyruvate et sur lactate de soude. *Ann. Inst. Pasteur*, **80**, 395.

Ware, G. C., Childs, E. & Smith, H. M. (1955). The effect of salt concentration on the growth of bacteria in dilute nutrient solutions. *J. appl. Bact.* **18**, 446.

ZoBell, C. E. & Rittenberg, S. C. (1948). Sulfate-reducing bacteria in marine sediments. *J. mar. Res.* **7**, 602.

(*Received* 12 *March* 1957)

Thirst, M. L. (1957). *J. gen. Microbiol.* **17**, 390–395

Hippurate Hydrolysis in Klebsiella-Cloaca Classification

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SUMMARY: 169 strains of *Klebsiella pneumoniae* and 68 strains of *Cloaca cloacae* were used in an examination of Hajna & Damon's hippurate test and various modifications of it. The addition of a pH indicator (phenol red) to the medium enabled hydrolysis to be detected by a change of colour. Clear-cut distinction between *K. pneumoniae* and *C. cloacae* was not obtained with any method, but the indicator medium gave the best results and was simplest.

Hydrolysis of sodium hippurate was used by Hajna & Damon (1934) to confirm the distinction, based on gelatin liquefaction, between *Aerobacter aerogenes* and *A. cloacae*. With a chemically defined medium containing sodium hippurate as the sole organic source of carbon, hydrolysis was shown by growth and could be confirmed by precipitating the resulting benzoate with a ferric chloride reagent. Other methods of detecting the hydrolysis of hippurate are the use of a pyridine + copper sulphate reagent (Munch-Petersen, 1940), or the observation of an increase in pH value (Cowan, 1955). Although Hajna & Damon claimed reliable separation between *A. aerogenes* and *A. cloacae*, the use of their test has not been widely reported; consequently there is little further evidence about its value in distinguishing between these species. Recently there has been a tendency to separate *Aerobacter aerogenes* from *A. cloacae* and to place them in different groups or genera. Their long association in the same genus makes workers reluctant, without good evidence, to accept this separation; therefore the hippurate test was re-examined and a comparison was made between tests with Hajna & Damon's medium and Cowan's micromethod. In the course of this comparison modifications were made to obtain better correlation between the hippurate tests and other criteria used in distinguishing these groups.

METHODS

Organisms. The strains used were those kept in the National Collection of Type Cultures (NCTC). *Aerobacter aerogenes*, which is commonly found in water, has the same cultural characters and reactions as most strains of *Klebsiella pneumoniae* although some of the latter may be atypical in their inability to produce gas from glucose, and in their MR and VP reactions. However, in general the two species are so similar that most workers now regard *A. aerogenes* as a later synonym of *K. pneumoniae* (*Report*, 1954) the name used in this paper. Two other species of *Klebsiella* are recognized, *K. ozaenae* and *K. rhinoscleromatis*. When *A. aerogenes*, the type species of *Aerobacter*, is removed the genus ceases to exist and another name must be found for *A. cloacae*; I shall use the name *Cloaca cloacae*. The Aerobacter type of paracolon, or 32011 type of Stuart, Wheeler, Rustigian & Zimmerman (1943), is referred to as *Hafnia alvei*. Table 1 shows the characteristics which distinguish the various species.

Table 1. *Distinguishing characters of the Klebsiella-Cloaca-Hafnia groups*

Character	*Klebsiella rhino-scleromatis*	*Klebsiella ozaenae*	*Klebsiella pneumoniae*	*Aerobacter aerogenes*	*Cloaca cloacae*	*Hafnia alvei*
Lysine decarboxylase	−	−	+	nr	−	+
Ornithine decarboxylase	−	−	−	nr	+	+
Arginine decarboxylase	−	−	−	nr	+	−
Koser's citrate	−	+	+	+	+	+
Urease	−	d	+	+	−	−
Methyl-red	+	+	−	−	−	−
Voges-Proskauer	−	−	+	+	+	+
Gluconate	−	−	+	nr	+	+
Malonate	+	−	+	+	+	+
Motility	−	−	−	−	+	+
Gelatinase	−	−	−	−	(+)	−
Gas from glucose	−	d	+	+	+	+
Acid from lactose	−	(+)	+	+	d	−
Gas from glycerol	−	+	+	+	−	(+)

+ = positive in 1–2 days; (+) = weak or delayed positive; − = negative; d = different strains, each consistent in itself, give different reactions; nr = not recorded.

Media. The defined medium (HD) was prepared by Hajna & Damon's (1934) method and a modified form (A) was made up as follows: NaCl, 0·5 g.; $MgSO_4.7H_2O$, 0·02 g.; $NH_4H_2PO_4$, 0·1 g.; K_2HPO_4, 0·1 g.; sodium hippurate, 0·3 g.; agar (New Zealand), 1·0 g.; 4 % (w/v) aqueous phenol red, 0·25 ml.; water, 100 ml. The medium, used in the form of slopes, was not cleared, and the final pH value (6·8–7·0) needed little or no adjustment. Sterilization was by autoclaving at 15 lb./sq.in for 15 min.

Detection of hydrolysis. Media HD and A were inoculated lightly from an overnight culture on nutrient agar. Medium HD was incubated for 5 days at the optimum temperature of the organism; after this it was examined for growth and two methods of testing for hydrolysis were used: (1) the ferric chloride test described by Hajna & Damon (1934); (2) the pyridine + copper sulphate test with Zwikker's (1931) reagent described by Munch-Petersen (1940).

Medium A was inspected at intervals during an incubation period of 7 days. In 1–2 days sodium hippurate decomposition was shown by slight growth and a pink colour which increased with further incubation until the entire medium was alkaline. A few late positives (3–7 days) were found. A fairly heavy inoculum did not give false positive results, but a light inoculum was preferred since it did not obscure early signs of growth. The microtest was performed by Cowan's (1955) method with bromothymol blue as the indicator.

RESULTS

Hajna & Damon's original method was compared with the modified method (using medium A) and with the microtest, using as test organisms 200 strains of Klebsiella and 68 strains of Cloaca. Munch-Petersen's method was tested on a smaller number of strains (series a, Table 2). The results are summarized in Table 2, which shows that, on HD medium, the ferric chloride and pyridine + copper sulphate tests gave similar results, but that for the Klebsiella strains these reagents were not as sensitive indicators of hydrolysis as was the development of alkalinity. Of the two tests based on the observation of pH value, medium A and the microtest, both gave essentially similar results, but the microtest was positive with 13 Cloaca strains and thus appeared to be less satisfactory for distinguishing organisms of the two groups.

Table 2. *Comparison of tests for hydrolysis of hippurate*

		Series a				Series b		
Species and no. of strains tested in each series	Result	Medium HD		Alkalinity in		Medium HD	Alkalinity in	
		$FeCl_3$	Pyr.-$CuSO_4$	Medium A	Microtest	$FeCl_3$	Medium A	Microtest
Klebsiella pneumoniae:	+	108	105	126	120	6	6	6
a, 157; b, 12	−	49	52	31	37	6	6	6
Cloaca cloacae:	+	4	4	0	13	0	0	0
a, 45; b, 23	−	41	41	45	32	23	23	23
K. ozaenae:	+	0	0	0	4	0	0	0
a, 10; b, 6	−	19	19	19	15	6	6	6
K. rhinoscleromatis:	+	.	.	.	.	0	0	0
a, 0; b, 6	−	.	.	.	.	6	6	6
Hafnia alvei:	+	.	.	.	.	0	0	12
a, 0; b, 18	−	.	.	.	.	18	18	6

DISCUSSION

The ferric chloride test depends on the relative solubilities of benzoate and hippurate in a solution of a ferric salt; the final concentration of iron is critical and measurement of both the culture fluid and the reagent is required. Munch-Petersen's method, consisting of the addition of Zwikker's reagent to an acid ether extract of the culture fluid, does not require accurate measurements; on the other hand, the preparation of the extract may be difficult with slimy cultures as these form emulsions with ether. Bacterial slime may also affect the microtest because it cannot be adequately separated from the bacterial cells, and the resultant light and badly washed suspensions may give false results. Doubtful results may be confirmed by testing chromatographically for the glycine liberated by the hydrolysis. Medium A has the advantage that it is unaffected by slime and the progress of the reaction may be observed throughout the test period. In contrast, both the tests on HD medium require the addition of reagents to the culture, which rules out repeated tests on the same culture.

The value of hippurate hydrolysis for distinguishing different species was assessed on medium A which gave sharper distinction than the microtest. *Cloaca cloacae* (68 strains), *Klebsiella ozaenae* (25), *K. rhinoscleromatis* (6) and *Hafnia alvei* (18) were consistently hippurate-negative, but *K. pneumoniae*

Table 3. *Comparison of hippurate reaction with other biochemical reactions and serotype of* Klebsiella pneumoniae *strains*

	Reaction										No. of strains and hippurate reaction		
Serotype	Lysine decarboxylase	Citrate	Urease	MR	VP	Gluconate	Malonate	Møller's KCN (1954)	Indole	Gas from glucose	+	(+)	−
1	+	+	+	−	+	+	+	+	−	+	0	0	3
	−	**−**	+	−	+	+	**−**	+	−	**−**	0	0	1
	+	**−**	+	−	+	+	+	+	−	**−**	0	1	1
	+	+	+	**+**	**−**	+	+	+	−	+	0	2	0
	+	+	+	**+**	**−**	+	+	+	−	**−**	0	1	0
	+	+	+	−	+	+	**−**	+	−	+	0	1	1
	+	+	+	−	+	+	+	+	−	**−**	0	0	1
2	+	+	+	−	+	+	+	+	−	+	13	0	0
	−	**−**	+	−	+	+	+	+	−	+	1	0	0
	+	**−**	+	−	+	+	+	+	−	+	0	0	1
	+	**−**	+	−	+	+	+	+	−	**−**	1	0	2
	+	+	+	−	+	+	**−**	+	−	**−**	1	0	0
	+	+	+	−	+	+	+	+	−	**−**	1	0	0
3	+	+	+	**+**	**−**	**−**	+	**−**	−	+	0	6	0
	+	+	+	**+**	**−**	+	+	**−**	−	+	0	1	0
	+	+	+	**+**	**−**	**−**	+	+	−	+	0	1	0
4	+	+	+	−	+	+	+	+	−	+	2	0	1
	+	**−**	+	−	+	+	+	+	−	+	1	0	0
5	+	**−**	+	−	+	+	+	+	−	+	1	0	0
	+	+	+	**+**	**−**	+	+	+	−	+	1	0	0
6	+	+	+	−	+	+	+	+	−	+	0	0	1
	+	**−**	+	−	+	+	+	+	−	+	3	3	0
7–69	+	+	+	−	+	+	+	+	−	+	45	2	6
	−	+	+	−	+	+	+	+	−	+	0	1	0
	+	**−**	+	−	+	+	+	+	−	+	1	0	0
	+	+	**−**	−	+	+	+	+	−	+	3	0	0
	+	+	+	**+**	**−**	+	**−**	+	**+**	+	0	1	0
	+	+	+	−	+	+	**−**	+	−	+	1	0	0
	+	+	+	−	+	+	+	+	**+**	+	4	0	2
Not known	+	+	+	−	+	+	+	+	−	+	15	3	6
	+	**−**	+	−	+	+	+	+	−	+	0	0	1
	+	+	**−**	−	+	+	+	+	−	+	4	1	2
	+	+	+	**+**	**−**	+	+	+	−	+	1	0	1
	+	+	+	−	+	+	**−**	+	−	−	0	0	1
	+	+	+	−	+	+	+	+	−	**−**	0	0	1
	+	+	+	−	+	+	+	+	**+**	+	9	0	5

Reactions in heavy type are atypical of the species.

(169) did not have the uniform behaviour which Hajna & Damon found in *Aerobacter aerogenes*. Hajna & Damon, working only with strains labelled *A. aerogenes*, excluded from their series strains which were anaerogenic or whose IMViC reactions were atypical. The species *K. pneumoniae* is not homogeneous, either culturally or serologically, and an attempt was made to see whether the varying hippurate reactions could be correlated with other atypical biochemical reactions, or with the serotype where this was known (Table 3)

In serotype 1 only 3 strains were biochemically typical of *Klebsiella pneumoniae* (Table 1) and these were hippurate-negative. Other strains of the serotype were either negative or late positives in the hippurate test; these were atypical in MR, VP, citrate, and/or malonate reactions, and some were anaerogenic (see Table 3). Serotype 3 was unusual in that all members had MR+ VP-reactions and hydrolysed hippurate in 3–5 days; the majority were gluconate-negative and KCN-negative (Møller, 1954). Only 4 strains of *K. rhinoscleromatis* had been serotyped; these were of type 3 and all were KCN-positive and hippurate-negative. Serotypes 4, 5 and 6 each included some *K. ozaenae* strains (hippurate-negative), and most of the NCTC serotype 4 strains were of this species. Neither of the *K. pneumoniae* strains in serotype 5 had reactions strictly typical of the species. In serotype 6 the hippurate and citrate reactions were inversely correlated. The majority of the strains in serotype 2 were hippurate-positive. In contrast to serotype 6, all the hippurate-negatives of serotype 2 were also citrate-negative. With three exceptions, serotypes 7 to 69 were each represented by a single strain, and 58 of these 66 strains were hippurate-positive. Seven members of this large group gave atypical biochemical reactions, but the divergent strains all hydrolysed hippurate. In general, the inability of strains of *K. pneumoniae* to hydrolyse hippurate could not be associated with any other atypical biochemical reaction.

It is thus evident that, except in serotype 3, the hydrolysis of sodium hippurate cannot be correlated with either the serological or biochemical reactions of *Klebsiella pneumoniae*, and that the test will not distinguish absolutely between this species and *Cloaca cloacae* and other species of *Klebsiella*. That this conclusion might have been expected, was implied by the absence from the literature of any reference to the test after Hajna & Damon's original report; however, it may be of some use for confirming the identity of strains such as those occurring in serotype 3 whose gluconate, MR, VP and cyanide reactions set them apart as a biochemically atypical group.

REFERENCES

Cowan, S. T. (1955). Biochemical tests for bacterial characterisation. 1. Hippurate test. *Int. Bull. bact. Nom. Tax.* **5**, 97.

Hajna, A. A. & Damon, S. R. (1934). Differentiation of *Aerobacter aerogenes* and *A. cloacae* on the basis of the hydrolysis of sodium hippurate. *Amer. J. Hyg.* **19**, 545.

Møller, V. (1954). Diagnostic use of the Braun KCN test within the Enterobacteriaceae. *Acta path. microbiol. scand.* **34**, 115.

MUNCH-PETERSEN, E. (1940). Studies on bovine mastitis. 1. Study of an experimental herd. *Bull. Coun. sci. industr. Res. Aust.* **34**, 96.

Report (1954). The Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Microbiologists. Reports on the groups: Salmonella, Shigella, Arizona, Escherichia, Klebsiella (Aerogenes, Aerobacter), Providence (29911 of Stuart *et al.*). *Int. Bull. bact. Nom. Tax.* **4**, 47.

STUART, C. A., WHEELER, K. M., RUSTIGIAN, R. & ZIMMERMAN, A. (1943). Biochemical and antigenic relationships of the paracolon bacteria. *J. Bact.* **45**, 101.

ZWIKKER, J. J. L. (1931). Het aantoonen en het afzonderen van barbitalen bij het taxicologisch onderzoek. *Pharm. Weekbl.* **68**, 975.

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Thirst, M. L. (1957). *J. gen. Microbiol.* 17, 396–400

Gelatin Liquefaction: a Microtest

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SUMMARY: A slide microtest for gelatin liquefaction is described. Members of the genera *Cloaca*, *Bacillus* and *Clostridium* were tested with varying results. A limitation of the method is its ability to detect only ready-formed enzyme. The main advantage is that small amounts of culture are needed and the organisms may be grown under any selected conditions.

Gelatinase activity in bacteria is detected by methods which often require the progeny of a small inoculum to liquefy relatively huge amounts of substrate at a temperature unfavourable to the growth of the organism. Lewert & Lee (1954), investigating the gelatinase of Helminth larvae, used a thin film of gelatin on a glass slide as substrate, liquefaction (hydrolysis) being shown by clear areas on the fixed and stained slide. The method required very small amounts of material and was quick, so that its possible application as a bacteriological test was considered.

METHODS

Strains from the National Collection of Type Cultures were used as test organisms; the main genera examined were *Cloaca* and *Bacillus*.

Gelatin slides. Clean dry microscope slides were dipped in a melted 5 % (w/v) aqueous solution of gelatin at 37–40°, the excess liquid was drained and the slides stood on end to dry in the air; this left a thin coating of gelatin on the slides. The prepared slides were stored in a Petri dish in the refrigerator. The stock solution of 5 % gelatin could be stored at 4° with 0·1 % (w/v) phenol added as a preservative; the presence of phenol did not affect the gelatinase activity.

Procedure. Drops (*c.* 0·01 ml.) of the sample, usually a broth culture or a heavy suspension made from an agar slope culture grown at the optimal temperature for the organism were placed on the slide together with a suitable control such as uninoculated broth or water. The slide was incubated at 22° for 2–20 hr. in a Petri dish containing a strip of moist filter-paper to minimize evaporation from the drops. As the drops spread when enzyme activity was great, about 8 samples/slide was a convenient number. The extreme ends of the slide were not used because the gelatin drained slightly downwards while the slides were drying. After incubation the gelatin was hardened and the culture killed by immersion in a mixture of 1 vol. formalin (40 % formaldehyde) and 9 vol. saturated aqueous mercuric chloride for 20–30 min.; the slides were then washed in water and stained with any convenient protein stain, usually dilute (1/100) carbol fuchsin. Where hydrolysis had occurred clear areas indicating gaps in the gelatin film were seen (Fig. 1). For rough quantitative work serial twofold dilutions were made and spotted in order of dilution; for qualitative tests one drop of each culture was used.

Parallel tube liquefaction tests were carried out in stab cultures in nutrient gelatin (containing gelatin, 12%, w/v; Lemco, 1%; NaCl, 0·5%; Evans peptone, 1%) incubated at 22° and read daily for 30 days.

RESULTS

Effect of incubation time

The sensitivity of the method increased with the time of incubation. Serial twofold dilutions in water of a 24 hr. broth culture of a strain of *Chromobacterium prodigiosum* (NCTC 1377) were incubated on slides for 2, 5 and 21 hr., then fixed and stained. After 2 hr. a clear space corresponded to dilution 1/2; at 5 hr. 1/8 was clear; after 21 hr. clearance occurred at 1/512 and partial clearance at 1/1024; the control (water) was coloured (Fig. 1).

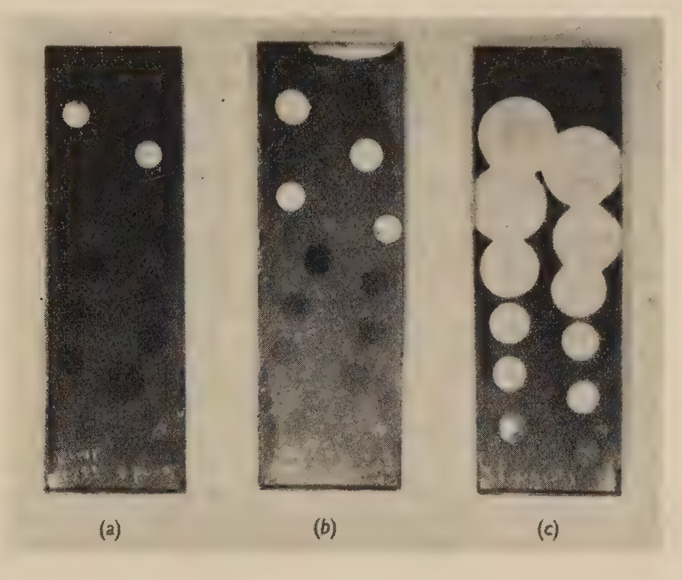

Fig. 1. Effect of incubation time on the sensitivity of the slide test. Lower right of each slide: water. Serial twofold dilutions in water of broth culture of NCTC 1377 placed on a zig-zag line from the upper left to the lower left of each slide. Slides incubated at 22° for (*a*) 2 hr., (*b*) 5 hr., (*c*) 21 hr.

Effect of hydrogen ion concentration and toluene

Since calcium ions are known to enhance gelatinase activity (Haines, 1931) calcium-binding buffer constituents such as citrate and phosphate could not be used; therefore 0·2 N-borate and acetate, and 0·2 M-ethylenediaminedihydrochloride buffers were used in a pH range of 2–9. The test organism was a strain of *Bacillus subtilis* (NCTC 6276) and heavy suspensions in buffer of different pH values were made from an overnight culture on Lemco agar. After 18 hr.

at 22° clearing had occurred in the range pH 5·6–7·7, and partial clearing at pH 5·0, 8·0 and 8·6. Serial dilutions were made in buffers at pH 6·0, 6·8, 7·2 and 7·7 and in distilled water; these were spotted on to slides which were incubated for 18 hr. before being fixed and stained. Clearing occurred at dilution 1/8 in water and in buffers at pH 6·0, 6·8 and 7·2, and at 1/4 in the pH 7·7 buffer. Thus for the gelatinase of NCTC 6276 the optimum pH appeared to be in the range 6·0–7·2, but the unbuffered suspension was as active as the buffered suspensions.

Treatment of the bacterial suspension with toluene for 1–2 hr. before testing did not enhance the gelatinase activity of 3 strains of *Cloaca cloacae* and 1 strain of *Bacillus coagulans*.

Tests on Cloaca cloacae

Sixty-two strains of *Cloaca cloacae* were tested as heavy suspensions from overnight Lemco agar cultures, the slides being incubated for 18–21 hr. The results, together with those of the routine gelatin stab tests, are summarized in Table 1. It can be seen that strains which liquefied gelatin stabs most slowly were the least likely to give positive reactions on slides. Two serum-digesting strains which were included in the series both liquefied gelatin stabs in 3 days and were positive in the slide test.

Table 1. *Gelatinase tests on strains of* Cloaca cloacae

Days to liquefaction in gelatin stab	No. of strains and slide reaction	
	+	−
1–5	14	0
6–10	4	2
11–13	1	4
14–20	2	14
21–30	3	5
Negative at 30 days	0	13

Slide tests and stab culture tests on 152 strains of *Klebsiella* spp. and 31 strains of the Alkalescens-dispar group were all negative. The slide tests for this series were done on suspensions prepared from 18–20 hr. Lemco agar slopes.

Tests on Bacillus *species*

Strains from 12 species of *Bacillus* (*B. alvei*, 5; *B. brevis*, 1; *B. cereus*, 5; *B. circulans*, 4; *B. coagulans*, 2; *B. licheniformis*, 4; *B. lentus*, 1; *B. macerans*, 1; *B. megaterium*, 2; *B. pumilus*, 6; *B. sphaericus*, 1; and *B. subtilis*, 14) were tested as unwashed suspensions from overnight Lemco agar slopes. The results (Table 2) do not show the same relation between slide reaction and time to liquefaction in stabs as was found with *Cloaca cloacae*. An attempt to correlate gelatinase activity with serum digestion showed that a higher proportion of slide-test positives occurred among the serum-digesting strains than among the non-digesting (serum-negatives), and the majority of serum-negative, slide-positive strains were able to liquefy gelatin stabs within 10 days.

Table 2. *Gelatinase tests on members of the* Bacillus *group*

Days to liquefaction in gelatin stab	No. of strains and			
	Slide reaction		Serum digestion	
			+	–
1–5	+	10	6	4
	–	0	0	0
6–10	+	10	6	4
	–	3	3	0
11–13	+	4	3	1
	–	1	0	1
14–20	+	5	4	1
	–	1	1	0
21–30	+	4	2	2
	–	4	0	4
Negative at 30 days	+	0	0	0
	–	4	1	3

Tests on Clostridium *species*

Tests on a few strains of *Clostridium* spp. were carried out with drops from cultures grown for 1–4 days in Robertson's meat broth; two 8-day cultures were also tested (*C. tetanomorphum* and *C. sphenoides*). The results are shown in Table 3. *C. welchii* was particularly active, 5 of the 6 strains tested giving positive slide reactions within 135 min. The stab tests on Clostridium strains were incubated under anaerobic conditions at 37° and were read after refrigeration.

Table 3. *Gelatinase tests on species of* Clostridium

	No. of strains and reaction in			
	Tube at 37°		Slide test	
Species	+	–	+	–
C. welchii type A	6	0	6	0
C. oedematiens type A	2	0	(2)	0
C. oedematiens type B	2	0	(1)	1
C. sporogenes	2	0	1, (1)	0
C. tetanomorphum	0	2	0	2
C. botulinum	1	0	1	0
C. sphenoides	0	1	0	1
C. putrefaciens	0	1	0	1

( ) indicates a weak reaction.

DISCUSSION

Among the limited number of strains tested, gelatinase activity as shown by the slide and stab culture tests was roughly parallel, and the proportion of strains showing a positive result in the slide test was greatest among those strains which most quickly liquefied gelatin in stab cultures; this relationship was more marked in *Cloaca cloacae* than in the *Bacillus* group. Many of the

Bacillus strains were able to digest serum, and the majority of these were gelatin-slide positive irrespective of the stab-liquefaction time; with the serum-negative strains the activity on gelatin slides decreased in parallel with that of the stab cultures in the same way as *C. cloacae* (serum-negative). This suggests that the serum-digesting enzymes of *Bacillus* species may reinforce the gelatinase activity in some strains but are not entirely responsible for it.

The results in Tables 1 and 2 show that the serum-negative strains which liquefied gelatin slowly did not give positive slide tests. Conditions on the slides do not favour the growth of organisms, therefore only enzymes which are present in appreciable amounts at the time of testing are detected, and slight or 'adaptive' activity does not give positive results. Attempts to make the test more sensitive by using preformalized slides at a higher temperature on the lines of the gelatinase test of Kohn (1953) were not successful. Within the limitations outlined, however, the gelatin slide technique may be of use as a bacteriological test, particularly for anaerobes, since these react under the aerobic conditions of the test.

REFERENCES

HAINES, R. B. (1931). The formation of bacterial proteases, especially in synthetic media. *Biochem. J.* **25**, 1851.

KOHN, J. (1953). Preliminary report on a new gelatin liquefaction method. *J. clin. Path.* **6**, 249.

LEWERT, R. M. & LEE, C. L. (1954). Studies on the passage of helminth larvae through host tissues. I. Histochemical studies on the extracellular changes caused by the penetrating larvae. II. Enzymatic activity of larvae *in vitro* and *in vivo*. *J. infect. Dis.* **95**, 13.

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Quantitative Analysis of the Gram Reaction

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SUMMARY: The crystal violet and iodine uptake by suspensions of *Staphylococcus aureus* and *Escherichia coli* was determined. It was found that equal amounts of intracellular dye-iodine complex were formed in these micro-organisms. The molar ratio dye to iodine was 1:2. The extractability of this complex with methanol and ethanol was compared with the relative solubility of dye-iodine precipitates. Results with *E. coli* suggest that the solvents pass freely into the cells and decolorize them after dissociation of the dye-iodine complex. Gram-positive micro-organisms may be arranged in a number of groups according to their extractability pattern with regard to the complex. These patterns suggest the presence of interaction between the cells and ethanol. This interaction may be located at the cell wall.

The elucidation of the mechanism underlying the Gram reaction seems to be complicated by a lack of quantitative information on the successive reactions leading to differentiation. The uptake of primary stain by bacterial suspensions has been studied in some detail, but the results are divergent. Moreover, the suspensions used were, with a few exceptions, killed by heat or fixatives which are likely to have affected the staining properties. The interpretation of the effects of iodine treatment and decolorizing agents is largely based on qualitative data obtained with heat-fixed smears; as appears from the careful compilation of the literature by Bartholomew & Mittwer (1952), these data have thus far given little information on the mechanisms involved. The object of the present work was to establish essential features of Gram-positivity and Gram-negativity. Quantitative data were collected on primary stain and iodine uptake by bacterial suspensions. The effects of fixation and of decolorizer concentration on the Gram reaction were also studied. It was found that suspensions could easily be differentiated without counter-stain; this step was therefore omitted in the procedure which consisted in staining with crystal violet, treatment with iodine and differentiation with ethanol.

METHODS

Organisms. The strains used included the Gram-positive species *Staphylococcus aureus, Streptococcus faecalis, S. lactis, Diplococcus pneumoniae* type II, *Lactobacillus arabinosus, L. plantarum, Bacillus cereus, B. subtilis, Clostridium welchii* type A, *Saccharomyces cerevisiae*; Gram-negative organisms included *Escherichia coli, Aerobacter aerogenes, Paracolobactrum* sp., *Pseudomonas fluorescens, Salmonella enteritidis, Serratia marcescens.*

Culture media. Staphylococcus aureus, Streptococcus faecalis, S. lactis, Bacillus cereus, B. subtilis and the Gram-negative rods were grown in a medium containing 1 % (w/v) Difco peptone, 0·3 % Difco meat extract, 0·5 % NaCl and 1 % glucose. Shake cultures were incubated for 16 hr. at 32°. *Diplococcus pneumoniae* was cultivated for 16 hr. at 37° in beef heart infusion broth containing 1 % Difco peptone, 0·5 % NaCl and 0·1 % glucose. *Clostridium welchii* was grown in a cooked-meat medium prepared from minced horse meat; cultures were incubated for 16 hr. at 37°. Stephenson & Rowatt medium (1947) was used for lactobacilli, which were cultivated for 48 hr. at 25°.

Bacterial suspensions. After incubation, organisms were centrifuged down, washed with distilled water and resuspended in distilled water. *Saccharomyces cerevisiae* suspensions were prepared from fresh commercial baker's yeast. The final aqueous suspensions were adjusted to a concentration of *c.* 8·5 mg. dry wt./ml. with the aid of a nephelometer (Evans Electroselenium Ltd.; EEL) and dry weight/turbidity reference curves. The suspensions were adjusted to pH 7·1–7·2, unless otherwise stated.

Dry weight and total nitrogen determinations. Dry weights were obtained by drying 1 ml. samples of the suspensions at 110° to constant weight. Total N determinations were performed by the Kjeldahl technique.

Gram reaction. Crystal violet (Gurr, Michrome brand, purity *c.* 85 %) was used as the primary stain. The solution in distilled water contained 0·96 mg. dye/ml., corresponding to 0·82 mg. crystal violet and 0·745 mg. crystal violet cation; stock solutions were adjusted to pH 7·1. Gram's iodine was prepared by dissolving 1 g. iodine and 2 g. potassium iodide in a small volume of distilled water; the resulting solution was diluted to a final volume of 300 ml. In the routine Gram-reaction procedure with bacterial suspensions, 96 % (v/v) ethanol in water was used for differentiation.

The primary stain uptake was determined as follows. Two ml. of the crystal violet solution were added to different amounts of bacterial suspension to a total volume of 5 ml. The mixtures were left for 10 min. at room temperature and centrifuged at 3000 rev./min. for 10 min. The dye concentration of the supernatant fluid was estimated by measuring the extinction of suitable dilutions in 2 % (w/v) of dipotassium phosphate by a Gallenkamp direct reading photo-electric colorimeter with an Ilford 625 filter and comparing with a calibration curve of the dye solution. The dye uptake of the organisms was computed from the difference in dye concentration between the original staining solution and the supernatant fluid. For the determination of the retention of absorbed dye, the stained sediments were suspended in 5 ml. of distilled water or the 96 % (v/v) ethanol solution. One wash with water and three washes with the ethanol solution were sufficient, since further washing did not remove any more dye. The sediments after washing with water were resuspended in 3 ml. distilled water and mixed with 2 ml. iodine solution. The mixtures were left for 10 min. at room temperature and centrifuged. The sediments were washed three times with 5 ml. of the ethanol solution; the dye concentrations in the ethanol washes were determined after addition of one drop of 0·1 N-$Na_2S_2O_3$ solution to the dilution of supernatant fluid in phosphate solution in order to reduce iodine. In a small series of comparative experiments absolute methanol instead of ethanol was used for differentiation.

Analysis of crystal violet-iodine complexes. The composition of precipitates resulting from the interaction of crystal violet and iodine solutions was determined by measuring crystal violet and iodine concentrations in the supernatant fluid obtained by centrifugation of the mixtures.

Graded amounts of the iodine solution used in the Gram reaction were added to a series of centrifuge tubes containing 2 ml. crystal violet solution (~ 1·49 mg. dye-cation); the total volume was 5 ml. The tubes were left at room temperature for 10 min. and centrifuged for 10 min. at 6000 rev./min. The supernatant fluid was analysed for crystal violet in the usual way; free iodine was titrated with 0·01 N-$Na_2S_2O_3$ solution.

Iodine uptake by bacterial suspensions stained with crystal violet. To a series of centrifuge tubes containing 1 ml. bacterial suspension graded amounts of Gram's iodine solution were added. Free iodine in the supernatant fluids obtained by centrifugation for 10 min. at 6000 rev./min. was titrated with a 0·01 N-$Na_2S_2O_3$ solution. The same procedure was applied to suspensions (1 ml.) stained with crystal violet and washed with distilled water. The amount of crystal violet taken up by the suspensions was varied by using crystal violet solutions of different strength. The values for free iodine at various concentrations of iodine in excess obtained with stained and control suspensions were plotted against amounts of iodine added to the suspensions. From the resulting curves the difference between iodine uptake by stained and control suspensions was calculated.

Effect of ethanol concentration on the retention of crystal violet and crystal violet-iodine complexes. The retention of crystal violet was determined by washing stained sediments three times with 5 ml. of 10–100 % (v/v) ethanol solutions in water and analysing the supernatant fluids for crystal violet. For the determination of the retention of the complex between crystal violet and iodine, the gram reaction as outlined before was performed in a series of centrifuge tubes up to the decolorizing step. The washed sediments were resuspended in 5 ml. of 50–100 % (v/v) ethanol in water; three washes with the ethanol solution were made. The dye concentration in the supernatant fluids was determined in the usual way; 1 drop of a 0·1 N-$Na_2S_2O_3$ solution was added to the dilution in dipotassium phosphate solution.

Relative solubility of the crystal violet-iodine complex in alcohol. The extractability of the crystal violet-iodine complex from bacteria was compared with the relative solubility of this complex in 50–100 % (v/v) ethanol or methanol solutions in water. Quantities of crystal violet-iodine precipitate, approximately equal to the amount of dye-iodine complex formed in Gram-stained suspensions and containing 1·49 mg. dye-cation, were washed with distilled water and resuspended in 5 ml. portions of 50–100 % (v/v) alcohol in water solutions; washing was repeated three times and the amounts of crystal violet dissolved were determined as indicated before. The solubility figures thus obtained would not necessarily agree with those resulting from standard procedures.

RESULTS

Influence of pH *value on crystal violet uptake*

Fig. 1 illustrates the results obtained with *Staphylococcus aureus* and *Escherichia coli* suspensions and crystal violet solutions adjusted at pH values varying from 2 to 9. The crystal violet uptake by both *S. aureus* and *E. coli* suspensions was greatly influenced by the pH value and reached a maximum at pH 7–8. Suspensions and crystal violet solutions used in the Gram reaction were henceforth adjusted to pH 7·1–7·2.

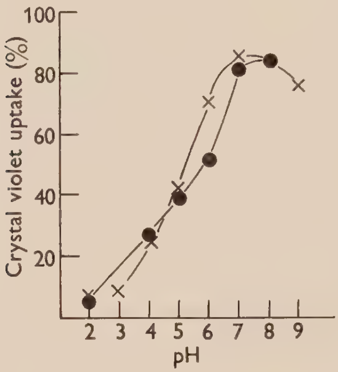

Fig. 1

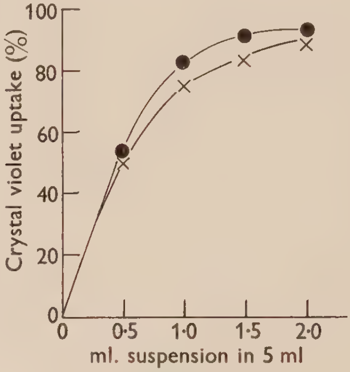

Fig. 2

Fig. 1. Influence of pH value on the crystal violet uptake by suspensions of *Staphylococcus aureus* (●) and *Escherichia coli* (×).

Fig. 2. Effect of the concentration of organisms on the crystal violet uptake by suspensions of *Staphylococcus aureus* (●) and *Escherichia coli* (×).

Maximum of crystal violet uptake

When a bacterial suspension was added to a given volume (2 ml.) of a crystal violet solution, the crystal violet uptake increased with the amount of suspension used; it reached a maximum of approximately 90 % (Fig. 2). The results presented in Fig. 2 are mean values obtained with 12 *Staphylococcus aureus* and *Escherichia coli* suspensions. They show a striking similarity between a Gram-positive and a Gram-negative organism in the pattern of crystal violet uptake. The crystal violet uptake by 0·5 ml. of the suspensions was not increased by doubling or tripling the strength of the crystal violet solution. Data concerning the maximal crystal violet uptake are summarized in Table 1. It is concluded from these data that *S. aureus* and *E. coli* absorb comparable amounts of crystal violet at pH 7·1.

Retention of crystal violet

On washing the stained organisms with distilled water only small amounts of crystal violet were found in the supernatant fluid after centrifugation; with 96 % (v/v) ethanol in water, however, the bulk of dye was extracted (Table 2). It will be noted that the ethanol solutions decolorized Gram-positive and Gram-negative cells to the same extent.

Table 1. *Crystal violet uptake by suspensions of* Staphylococcus aureus *and* Escherichia coli

	S. aureus	*E. coli*
Dry weight (mg./ml. suspension)	8·6 ± 0·17*(24)†	8·7 ± 0·23 (24)
Total N (% of dry weight)	11·6 ± 0·27 (7)	11·7 ± 0·26 (6)
Crystal violet uptake:		
By 0·5 ml. of suspension (% of initial concentration)	54 ± 2·4 (12)	50 ± 3·7 (12)
mg. dye cation/mg. dry weight	0·19	0·17
mg. dye cation/mg. N	1·64	1·45

* Standard error.
† Number of determinations.

Effect of iodine treatment on crystal violet retention

Suspensions of *Staphylococcus aureus* and *Escherichia coli* were subjected to the Gram procedure up to the stage of treatment with the counter stain and omitting, as usual, the fixation step. The results are summarized in Table 3. They clearly indicate that a Gram differentiation can be obtained with unfixed bacterial suspensions.

Table 2. *Crystal violet removal from Gram-positive and Gram-negative micro-organisms by water or 96 % (v/v) ethanol in water*

	Crystal violet taken up (mg.)	Crystal violet removed (%)	
		Water	Ethanol
Staphylococcus aureus	1·22	7 (15)*	82 (8)
other positive organisms†	1·26	10 (9)	87 (5)
Escherichia coli	1·22	12 (16)	85 (8)
other negative organisms‡	1·16	14 (10)	88 (6)

* Number of determinations.

† Viz. *Streptococcus faecalis, Diplococcus pneumoniae, Lactobacillus plantarum, Bacillus subtilis* and *Clostridium welchii.*

‡ Viz. *Paracolobactrum* sp., *Aerobacter aerogenes, Salmonella enteritidis, Pseudomonas fluorescens* and *Serratia marcescens.*

The residual colour of the suspensions corresponded to that of heat-fixed smears stained by the usual Gram procedure (counter-staining omitted). From Tables 2 and 3 it is apparent that the iodine treatment is essential for differentiation.

In a small series of experiments to compare the decolorization by different alcohols, iodine-treated suspensions were washed with ethanol or methanol solutions. The results listed in Table 4 show that methanol decolorizes Gram-positive as well as Gram-negative organisms. This effect could not be attributed to a difference in the solubility of the crystal violet-iodine complex in these alcohols. As appears from Fig. 5, the complex dissolves more readily in ethanol than in methanol solutions.

Analysis of crystal violet-iodine complexes

On addition of iodine to crystal violet solutions a flocculent precipitate appears which can easily be removed by centrifugation; in the presence of an excess of iodine, however, a rather stable suspension is formed of small dye particles which are difficult to spin down. The supernatant fluids obtained on centrifugation of crystal violet-iodine mixtures prepared by adding varying quantities of Gram's iodine solution (3·18 mg. iodine/ml.) to constant volumes of a crystal violet solution (2 ml.) were analysed for crystal violet and free iodine. The results are indicated in Fig. 3, which shows that crystal violet was completely precipitated by 0·325 ml. iodine solution. The crystal violet solution contained 1·49 mg. dye cation which was precipitated by 0·325 × 3·18 mg. = 1·03 mg. iodine. The weight to weight ratio of crystal violet to iodine in the precipitate (1·49 : 1·03 = 1·44) indicates equimolarity. Thus, at the iodine concentration indicated the precipitate consists of equal numbers of crystal violet cations and iodine molecules. However, as may be seen from the results in Fig. 3, the iodine concentration could be doubled without iodine appearing in the supernatant fluid. The ratio of crystal violet cations to iodine molecules in the precipitate at this high iodine concentration is therefore 1 : 2. It thus appears that two species of crystal violet-iodine complexes are formed, their abundance in any precipitate depending on the ratio between the concentrations of dye and iodine in the original mixture.

Table 3. *Crystal violet removal from suspensions of* Staphylococcus aureus *and* Escherichia coli *treated with iodine and washed with* 96 % *(v/v) ethanol in water*

	Crystal violet taken up (mg.)	Crystal violet removed (%)
S. aureus (25)*	1·18	40
E coli (25)	1·21	97

* Number of determinations.

Table 4. *Crystal violet removal from suspensions treated with iodine and washed with methanol or* 96 % *(v/v) ethanol in water*

	Crystal violet taken up (mg.)	Crystal violet removed (%)	
		Methanol	Ethanol
Staphylococcus aureus (3)*	1·00	100	12
Bacillus subtilis (2)	1·22	75	35
B. cereus (2)	1·25	82	29
Escherichia coli (3)	1·06	100	97

* Number of determinations.

Crystal violet-iodine complex in bacteria

Determinations of iodine uptake showed that more iodine is taken up by stained than by unstained bacteria. By varying the amount of dye absorbed by the suspension a relationship between extra iodine uptake and dye absorbed could be demonstrated. This is shown in Fig. 4 which illustrates results obtained with *Staphylococcus aureus* and *Escherichia coli*. The mean ratio of crystal violet: extra iodine was 0·76; this value corresponds to one crystal violet-cation for two iodine molecules taken up, and agrees well with the dye-iodine proportion occurring in precipitates formed in the presence of an excess of iodine. It is clear from these results that iodine treatment of stained suspensions of a Gram-positive and a Gram-negative organism results in the formation of presumably identical dye-iodine complexes.

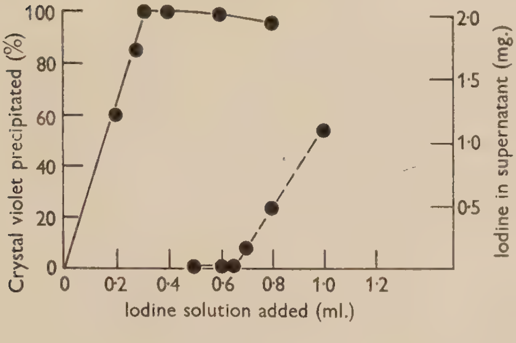

Fig. 3

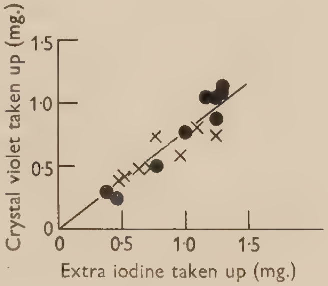

Fig. 4

Fig. 3. Crystal violet in precipitates (—) and free iodine (– – –) in supernatant fluids from mixtures of crystal violet and iodine solutions. The iodine solution (3·18 mg. iodine/ml.) was added to 2 ml. crystal violet solution (0·745 mg. crystal violet cation/ml.) and the mixtures centrifuged.

Fig. 4. Relation between crystal violet and extra iodine uptake by suspensions of *Staphylococcus aureus* (●) and *Escherichia coli* (×).

Effect of the ethanol concentration on the extractability of the crystal violet-iodine complex

It was concluded from the above results that the differences between the extractability of dye-iodine complexes from Gram-positive and Gram-negative organisms might be due to differences in the interaction of the cells with the solvent. Early in the literature reports were published showing that the addition of water to ethanol increases decolorization. This effect was studied in the present work. Suspensions of Gram-positive or Gram-negative organisms were stained with crystal violet and treated with iodine; the extractability of the dye-iodine complex by different concentrations of ethanol was determined in the usual way and compared with the relative solubility of dye-iodine precipitate in these solvents; this is illustrated in Fig. 5.

The mean extractability curves obtained with *Escherichia coli* and other Gram-negative organisms show that extraction of the dye complex by 80 to 100 % (v/v) ethanol in water is nearly complete (Fig. 6). There is a close correlation between the curves in Fig. 6 and the solubility curve of the dye-iodine precipitate (Fig. 5). This correlation suggests that ethanol passes freely in and out of these cells. Dissociation of the dye-iodine complex is followed by a distribution of the intracellular crystal violet over the total volume of ethanol solution, resulting in decolorization.

The results obtained with Gram-positive organisms were completely different (Fig. 7), and conformed to the solubility curve of the dye-iodine precipitate (Fig. 5) up to a certain concentration of ethanol. Beyond this critical concentration, which varies for different groups of Gram-positive organisms, a sharp decrease of extractability occurs. It is seen from the curves that organisms which are known to be strongly Gram-positive show low maxima of extractability.

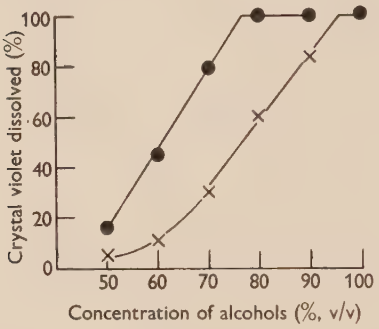

Fig. 5

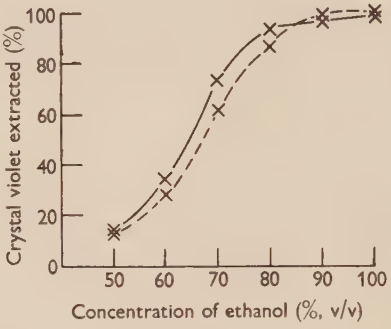

Fig. 6

Fig. 5. Relative solubility of crystal violet-iodine precipitates containing 1·49 mg. dye cation in 50–100 % (v/v) ethanol (●) or methanol (×) in water solutions. Precipitates were prepared in the presence of an excess of iodine; ratio of crystal violet cations: iodine molecules = 1 : 2 (see text).

Fig. 6. Effect of the ethanol concentration on the extractability of the crystal violet-iodine complex from *Escherichia coli* (×—×) and other Gram-negative organisms (×---×), viz. *Serratia marcescens*, *Aerobacter aerogenes* and *Paracolobactrum* sp.

In Fig. 8 maximum values of crystal violet extraction have been plotted against the corresponding ethanol concentrations. The distribution of the maxima along the relative solubility curve of the crystal violet-iodine precipitate suggests that the absolute value of a maximum for a given organism depends on the ethanol concentration at which it is reached. According to the strength of the ethanol solution required for maximal extraction the organisms studied may be arranged in the following sequence of Gram-positivity: yeast, spore-forming organisms, lactobacilli, micrococci, streptococci.

When the pretreatment with iodine of suspensions of *Staphylococcus aureus* and *Escherichia coli* was omitted the extraction pattern of the dye by varying concentrations of ethanol was similar for both organisms. Figure 9 shows the extractability of crystal violet and the crystal violet-iodine complex as a function of the ethanol concentration. The inability of high concentrations of ethanol to extract dye which it is suggested is a characteristic of Gram positivity, clearly depends on pretreatment with iodine.

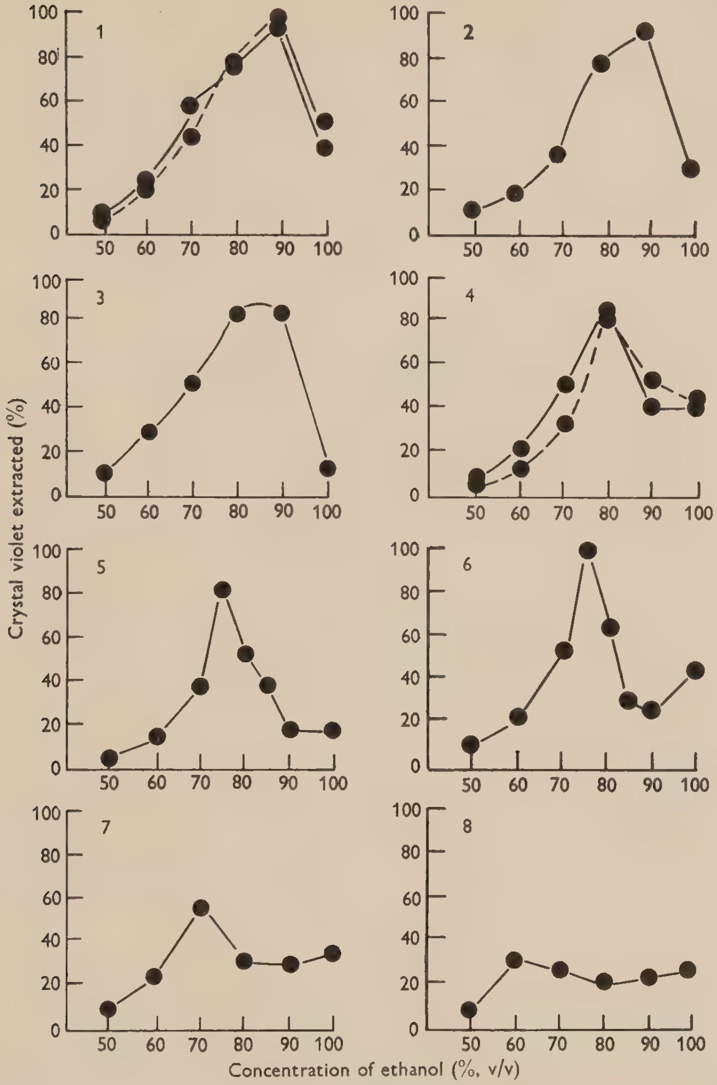

Fig. 7. Effect of ethanol concentration on the extractability of the crystal violet-iodine complex from Gram-positive micro-organisms. 1 = *Streptococcus faecalis* (——) and *S. lactis* (– – –); 2 = *Diplococcus pneumoniae*; 3 = *Staphylococcus aureus*; 4 = *Lactobacillus plantarum* (——); *L. arabinosus* (– – –); 5 = *Clostridium welchii*; 6 = *Bacillus cereus*; 7 = *B. subtilis*; 8 = *Saccharomyces cerevisiae.*

Gram reaction in fixed suspensions

From the above experiments it is apparent that perfect Gram differentiation may be obtained with native bacterial suspensions. It was therefore concluded that fixation plays no essential role in the Gram reaction. Nevertheless, fixation has definite effects on the primary stain uptake by suspensions. In a small series of experiments, suspensions of *Staphylococcus aureus* and

Escherichia coli were heated in a boiling-water bath for periods from 30 min. to 16 hr. This treatment, involving a considerable loss of cell material (consisting mainly of ribonucleic acid), leads to a decrease of the uptake of crystal violet. Washing with distilled water, however, restored the capacity to absorb dye of the heated organisms to that of the native suspension; Gram reactions on these washed suspensions gave normal results. The same applies to suspensions treated with 96% (v/v) ethanol-ether (1:1), a mixture used for the fixation of slide preparations. Fig. 10 illustrates the results of these experiments.

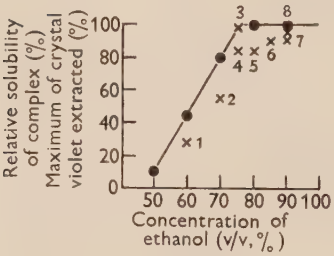

Fig. 8

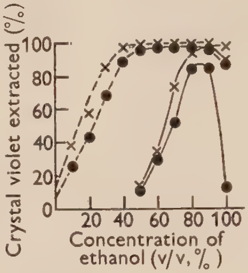

Fig. 9

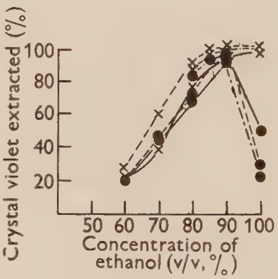

Fig. 10

Fig. 8. Diagram indicating the relationship between maximum values of crystal violet extraction and ethanol concentration. ●—●, relative solubility of crystal violet-iodine precipitate. 1 = *Saccharomyces cerevisiae*; 2 = *Bacillus subtilis*; 3 = *B. cereus*; 4 = *Clostridium welchii*; 5 = *Lactobacilli*; 6 = *Staphylococcus aureus*; 7 = *Diplococcus pneumoniae*; 8 = *Streptococci*.

Fig. 9. Extractability of crystal violet (– – –) and the crystal violet-iodine complex (——) from suspensions of *Staphylococcus aureus* (●) and *Escherichia coli* (×) by 10–100% (v/v) ethanol.

Fig. 10. Extractability of the crystal violet-iodine complex from fixed suspensions of *Staphylococcus aureus* (●) and *Escherichia coli* (×). ——, fixation at 100° for 30 min.; – – –, fixation at 100° for 60 min.; –·–·–, fixation with a 96% (v/v) ethanol-ether (1:1) mixture.

DISCUSSION

The results of our experiments on crystal violet uptake (Fig. 2 and Table 1) show that equal amounts of dye are taken up by standardized native suspensions of a Gram-positive and a Gram-negative organism. The values calculated as mg. dye cation/mg. dry weight or mg. bacterial N agree well with the data of Bartholomew & Finkelstein (1954) for heat-killed *Staphylococcus aureus* and *Escherichia coli*. From a few experiments Kennedy & Barbaro (1953) concluded that more crystal violet was taken up by Gram-positive than by Gram-negative organisms; a Gram-variable corynebacterium assumed an intermediate position. However, the figures reported by these authors (Barbaro & Kennedy, 1954) for the uptake of crystal violet are widely spread, according to them because of many variables. Finkelstein & Bartholomew (1956) drew attention to the decolorizing effect of the buffer solutions used by Barbaro & Kennedy and concluded that no correlation between Gram-character and crystal violet uptake exists when suspensions are washed with distilled water. As no difference in crystal violet uptake was found between

S. aureus and *E. coli* at various pH values (Fig. 1) it seems probable that the Stearn & Stearn concept (1924) which correlates Gram character with dye uptake capacity should be abandoned. This concept is essentially based on presumed differences between iso-electric points of Gram-positive and Gram-negative cells. These differences could not be demonstrated by electrophoresis (Harden & Harris, 1953).

A simple explanation of the effect of iodine on the extractability of crystal violet from Gram-positive and Gram-negative organisms by 96% (v/v) ethanol in water (Tables 2 and 3) might be a difference in the interaction between crystal violet and iodine in the two groups. This possibility, however, seems to be ruled out by the results of experiments on iodine uptake by stained suspensions; these showed that the dye-iodine complex in both *Staphylococcus aureus* and *Escherichia coli* contains two molecules of iodine/dye cation (Fig. 4) and corresponds to crystal violet-iodine precipitates formed in the presence of excess iodine (Fig. 3). The extractability of the dye-iodine complex from Gram-negative organisms seems to be merely a function of its solubility. The curves (Fig. 6) suggest that rapid penetration of ethanol occurs. The extractability from Gram-positive organisms however parallels the solubility only up to a critical concentration of ethanol. Beyond this concentration the extractability is considerably decreased (Fig. 7). This may be due to a decrease of penetration or to interference with the outward diffusion of the solvent. These processes were studied in heat-fixed smears stained with crystal violet and treated with iodine. In *S. aureus* smears immersed in 80% (v/v) ethanol in water solution decolorization was preceded by a colour change from black to blue resulting from dissociation of the dye-iodine complex. Immersion in absolute ethanol was not followed by colour changes, indicating that no dissociation occurred. It was concluded from this observation that in Gram-positive micro-organisms the penetration is impeded by high concentrations of ethanol.

Iodine treatment is essential for the demonstration of decreased penetration of the solvent. Fig. 9 illustrates the importance of iodine treatment and ethanol concentration in the Gram-differentiation. It is seen that comparable amounts of the dye-iodine complex are extracted from *Staphylococcus aureus* by 50% (v/v) aqueous and absolute ethanol. It may be concluded therefore that the final intra-cellular concentration of ethanol is *c.* 50% in both cases. The curves show that the extractability of crystal violet in the absence of iodine is nearly complete at this concentration of ethanol. Clearly, the function of iodine is to lower the solubility of the absorbed dye to a critical level at which it is very susceptible to the concentration of the solvent. This supposition finds support in the demonstration that suitability of basic dyes for the Gram reaction depends partly on the solubility of their iodine complexes in ethanol (Bartholomew & Mittwer, 1951). Specific effects of solvents however are important. Thus, methanol was found to decolorize both Gram-positive and Gram-negative organisms (Table 4) in spite of a lower solubility of the crystal violet-iodine precipitate in this solvent than in ethanol (Fig. 5).

The nature of the substrate in Gram-positive organisms which interacts in a characteristic way with ethanol and ethanol-water mixtures is unknown. It was concluded from experiments with fixed suspensions (Fig. 10) that this substrate is highly thermostable. Moreover, Gram-reactions produced normal results with suspensions of *Staphylococcus aureus* pretreated with solvents which were shown to decolorize *S. aureus* completely (methanol, 80 % (v/v) ethanol in water). It is therefore concluded that solvent interactions with Gram-positive material are reversible. They are likely to occur in the cell wall, known from early experiments (Burke & Barnes, 1929) to play a major role in the Gram reaction.

From these considerations it appears that a study of cell-wall properties and particularly of interactions with organic solvents will provide much more information on Gram-positivity than will attempts to isolate Gram-positive cell material. Crystal violet stains almost every type of cell and forms intracellular complexes with iodine which have not been shown to be associated with a particular cell component. The fact that this complex cannot easily be extracted from a small fraction of cell types may be related to the characteristic structure of their cell walls. Some of the prominent features of Gram-positive cell walls are their low lipid content (Salton, 1953) and the presence of glycerophosphate which may be part of a lipid-glycerophosphate-protein cell-wall component (Mitchell & Moyle, 1951, 1954). The effect of glycerophosphate removal on Gram-positivity which has been reported by Mitchell & Moyle (1950) remains to be studied in detail.

REFERENCES

BARBARO, J. F. & KENNEDY, E. R. (1954). A quantitative gram reaction. *J. Bact.* **67**, 603.

BARTHOLOMEW, J. W. & FINKELSTEIN, H. (1954). Crystal violet binding capacity and the gram reaction of bacterial cells. *J. Bact.* **67**, 689.

BARTHOLOMEW, J. W. & MITTWER, T. (1951). The mechanism of the Gram reaction. III. Solubility of dye-iodine precipitates and further studies on primary dye substitutes. *Stain Technol.* **26**, 231.

BARTHOLOMEW, J. W. & MITTWER, T. (1952). The Gram stain. *Bact. Rev.* **16**, 1.

BURKE, V. & BARNES, M. W. (1929). The cell wall and the Gram reaction. *J. Bact.* **18**, 69.

FINKELSTEIN, H. & BARTHOLOMEW, J. W. (1956). Influence of phosphate buffer on crystal violet uptake and retention by bacterial cells as related to the Gram reaction. *J. Bact.* **72**, 340.

HARDEN, V. P. & HARRIS, J. O. (1953). The isoelectric point of bacterial cells. *J. Bact.* **65**, 198.

KENNEDY, E. R. & BARBARO, J. F. (1953). Quantitative adsorption of crystal violet. *J. Bact.* **65**, 678.

MITCHELL P. & MOYLE, J. (1950). Occurrence of a phosphoric ester in certain bacteria: its relation to Gram staining and penicillin sensitivity. *Nature, Lond.* **166**, 218.

MITCHELL, P. & MOYLE, J. (1951). The glycerophospho-protein complex envelope of *Micrococcus pyogenes*. *J. gen. Microbiol.* **5**, 981.

MITCHELL, P. & MOYLE, J. (1954). The Gram reaction and cell composition: nucleic acids and other phosphate fractions. *J. gen. Microbiol.* **10**, 533.

SALTON, M. R. J. (1953). Studies of the bacterial cell wall. IV. The composition of the cell wall of some Gram-positive and Gram-negative bacteria. *Biochim. biophys. Acta*, **10**, 512.

STEARN, E. W. & STEARN, A. E. (1924). The chemical mechanism of bacterial behaviour. I. Behaviour towards dyes; factors controlling the Gram reaction. II. A new theory of the Gram reaction. *J. Bact.* **9**, 463, 479.

STEPHENSON, M. & ROWATT, E. (1947). The production of acetylcholine by a strain of *Lactobacillus plantarum*. *J. gen. Microbiol.* **1**, 279.

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Boyne, A. W., Eadie, J. M. & Raitt, K. (1957). *J. gen. Microbiol.* 17, 414–423

The Development and Testing of a Method of Counting Rumen Ciliate Protozoa

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SUMMARY: The limitations of methods previously used for counting rumen ciliates, and the value of developing a method of known accuracy, are discussed. Two counting-chambers, one based on the Sedgwick-Rafter cell, the other on the MacMaster eelworm-counting cell, were constructed and tested. A MacMaster-type cell was adopted. The preparations of suitable suspensions for counting organisms from washed suspensions and from rumen contents are described. The counting technique adopted was found to be reliable; its accuracy depends upon the number of organisms counted. In one sheep on a constant diet, day-to-day differences in rumen ciliate population were far greater than the differences found throughout the rumen at any one time, or the variations due to technique.

Many workers studying rumen protozoal fauna have given counts of ciliate protozoa in their results. Their object has most frequently been to compare ciliate populations after changes of diet in an attempt to correlate ciliate population with digestion in the animal (Ferber, 1928; Ferber & Winogradawa-Federowa, 1929; Mowry & Becker, 1930); the work of van der Wath & Myburgh (1941), Weller & Gray (1954), Moir (1951) and Williams & Moir (1951) had much the same objective. Hungate (1942) in the investigation of the division rate of his cultures of *Eudiplodinium* spp., illustrated the use of a counting method for *in vitro* studies. Gutierrez (1955) made biochemical examinations of the holotrich protozoa *in vitro*, and used counts made from these experiments and from whole rumen liquor in order to determine the contribution of the holotrichs to volatile fatty acid production *in vivo*. However, despite the significance frequently attached to the results very little attention seems to have been given to the development of a counting method of known reliability and there has been little consistency either in the methods used or in the results obtained. Oxford (1955) enumerated some of the difficulties involved in making accurate rumen ciliate counts, but also stressed the importance of being able to assess the total and differential population (e.g. holotrichs and various kinds of oligotrichs) in order to relate the contributions of particular ciliates to ruminant digestion. Adam (1951), after describing her method for counting horse intestinal ciliate protozoa, pointed out that she was unable to compare these results with total rumen ciliate counts because no really reliable results were available. Her most obvious criticism of the total rumen ciliate counts available was that there was no means of assessing how far the small rumen samples used were truly representative. It is clear that until a reliable method is found to determine with a known degree of accuracy the concentration of protozoa in a given rumen sample, no conclusions can be drawn as to the value of that sample as a representative of the rumen as a whole. Although it might eventually be found that in routine counts certain experimental refinements may be unnecessary in the light of differences between sheep on the same ration, it is first of all essential to know how much of the total variability encountered on any occasion is due to the method used. The present paper describes the development of a method, based on that of Adam (1951), by which counts of rumen ciliates both *in vitro* and from total rumen liquor, may be obtained, with known accuracy.

METHODS

Rumen ciliate protozoa have greater variation in size than protozoa from many other sources. Many counting methods, e.g. nephelometry, depend on the organisms being of uniform size. Previous workers have used counting chambers of standard design and most work on rumen ciliate counts has been done with haemocytometers (Ferber, 1928; Johnson, Hamilton, Robinson & Garey, 1944; Weller & Gray, 1954; Moir, 1951). These blood-cell counting chambers (e.g. Fuchs Rosenthal used by Ferber) are usually 0·2 mm. deep and, though accurate when counting blood corpuscles of *c.* 7 μ. diameter, would hardly be effective with organisms which may be as long as 200 μ. Although most of the rumen ciliates are small (<60 μ. long), if a true total and differential count is to be obtained another type of counting chamber is required.

Adam (1951, 1953) developed a method for counting horse intestinal ciliates, which also show a great variation in size, using the Sedgwick-Rafter cell previously found effective by Hall, Johnson & Loefer (1935) for counting pure suspensions of ciliates, and by Gilbert (1942) for counting phytoplankton varying in size from single to colonial forms. The Sedgwick-Rafter cell has the added advantage that the capacity is larger than that of a haemocytometer and it is therefore less subject to sampling error. It is noteworthy that Moir & Somers (1956) when counting rumen ciliates of sheep have recently turned from the use of a haemocytometer (Williams & Moir, 1951) to a modified form of Adam's technique, but no details are given.

Preparation of a Sedgwick-Rafter type of counting cell similar to that of Adam. A counting chamber of exactly 1 ml. capacity, based on that used by Adam (1951), was prepared with a 3 in. × 1·5 in. microscope slide as base. Strips of normal microscope slides exactly 1 mm. in thickness were firmly attached to it to give a chamber exactly 1 ml. in volume. A commercial cement 'Coaguline' (Kay Brothers Ltd., Stockport) was used to fix the strips, the layer required being so thin that it did not affect the dimensions. A large coverslip was carefully cut to cover the 20 × 50 mm. oblong cavity and to rest on the platform formed by the cut sections of slides.

The function of the Sedgwick-Rafter cell as used by Adam was to obtain a layer of suspended organisms 1 mm. deep, to allow them to sink to the base of the cell and then to be able to count random 1 mm.^{2} areas by the use of an eyepiece with an accurately marked area of 1 mm.^{2} when projected. In this way the organisms originally in 1 mm.^{3} of the fluid could be counted.

Preparation of a cell based on that used by Adam and the MacMaster eelworm *type of counting cell.* The second counting chamber prepared was like the MacMaster cell in design and was composed simply of a normal microscope slide firmly fixed 1 mm. above a large 3 in. × 1·5 in. slide, leaving a space into which the suspension was drawn and held in place by surface tension. Two platforms made from strips of microscope slides exactly 1 mm. thick were fixed 20 mm. apart on the base slide and the upper slide firmly attached to these. As before, 'Coaguline' was used as adhesive; this proved extremely firm and was unaffected by glycerol or frequent washing. A glass-pencil mark along the length of the upper slide allowed a regular check that no sagging had occurred. As microscope slides are optically level the only accurate measurement required was the 1 mm. thickness of the side platforms.

Advantages and disadvantages of the cells and the final technique of counting adopted. Note was taken of the warnings of Gilbert (1942) and of Adam (1951) that care is required to fill the slide with a fixed volume of fluid suspension; it was never possible to fill the Sedgwick-Rafter cell with exactly 1 ml. of suspension and place the coverslip in position without the formation of an air bubble. This was caused by the escape of fluid between the coverglass and the platform. The bubble could be eliminated if slightly more than 1 ml. of suspension was used, but as it was noted that only fluid escaped, there was in effect a certain straining action and the organisms tended to be more concentrated round the edge of the cell. A MacMaster type of cell was therefore constructed and counts made with it were tested statistically.

The MacMaster type of cell could be filled with just over 1 ml. of suspension from a pipette held in one position at the edge and, provided the suspension was suitable (see later), it was possible to avoid air bubbles at the enclosed edge and to obtain what appeared to be an even suspension of organisms. The ciliates were denser than the suspending fluid, and after about 5 min. had sunk to the base of the cell where they could be counted. A square micrometer eyepiece scale was calibrated to project an area of exactly 1 mm.^{2} on to the lower slide. The magnification used was limited by the necessity of having this 1 mm. square completely within the field of vision. This was best achieved with a × 6 eyepiece in the straight monocular body of an M-2000 microscope (Cooke, Troughton and Simms, York) using a $\frac{2}{3}$ in. achromatic objective. This gave a magnification of approximately × 60 with which the organisms could be distinguished even when obtained from whole rumen liquor. The vernier scale of the microscope stage very conveniently gave fixed positions on the slide: nineteen positions lengthwise and eleven from top to bottom with a slight space between each field. A table of random numbers was used to obtain random positions on the slide.

It was difficult to prepare suspensions of organisms from which samples could be removed to fill the counting chamber. One possible error which has been noted but not adequately compensated by previous workers is due to the rapid sedimentation of some of the rumen ciliates. The object was therefore to obtain a suspension of organisms fixed in a manner which left them recognizable and suspended in a fluid in which the viscosity greatly decreased the sedimentation rate but nevertheless allowed the organisms to settle in a few minutes on the base of the counting chamber. Ferber (1928), whose counts are the most generally quoted, suspended the organisms in water which, besides being non-viscous, rapidly causes bursting of the holotrichs. Weller & Gray (1954) used formalin, and Moir (1951) an iodine solution. Hungate (1942) and Gutierrez (1955) used suspensions in rumen fluid, and although thorough mixing was carried out Gutierrez made serial dilutions from the suspensions, and samples as small as 0·1 ml. were removed. As certain rumen ciliates settle even in the course of emptying a pipette, the chances of error are very real, so that the effect of consistency of rumen liquor on the protozoa population, mentioned by Mangold (1929), may be real, but may equally well be due to bias in sampling because of the effect of consistency on the rate of settling.

Preparation of washed suspensions of protozoa. Suspensions consisting largely of holotrich organisms separated by the method of Heald, Oxford & Sugden (1952) were used in testing various suspending media. These organisms when filled with storage material during the separation process were the most dense likely to be encountered. A series of suspensions in glycerol + buffer were tested (Adam, 1951). It was not found necessary to use a histological fixative since the glycerol rapidly killed the organisms and though it caused some distortion at first, within 5 min. the organisms regained their original shape. It had the added advantage of clearing the ciliates so that even well-filled holotrich organisms were clearly distinguished by the position of the mouth, and no stain was necessary. An approximately 50 % (v/v) mixture of glycerol and buffer proved the most effective.

Preparation of suspensions of protozoa from rumen liquor. It was necessary to separate the protozoa from the debris in such a way as to avoid loss or death of protozoa and at the same time to retain only particles of debris of comparatively small size. This is particularly important because in the large counting chamber, all the material in 1 $mm.^3$ is accumulated on an area of 1 $mm.^2$ and pieces of debris which might mask protozoa must therefore be avoided. Adam (1951) appears to be the only worker who has seriously attempted to separate the organisms from debris.

A series of straining materials and methods was tested including muslin (as used by Adam), bolting silk, and nylon, but eventually metal gauze soldered on to a cylindrical tin in place of the base was found most effective. Sieves of mesh 40, 80 and 120 were used in succession as described below. It was found essential to avoid copper in the sieve as the holotrichs were rapidly killed and disintegrated when copper, brass or monel metal gauze was employed. Instead, stainless steel gauze of woven wire cloth I.S.W.G. 41 (Baird and Tatlock, Chadwell Heath, Essex) was obtained and this was non-toxic.

The sample of rumen contents was passed through the coarse sieve into a 2 l. beaker containing some buffer (NaCl, 0·5 %; anhydrous sodium acetate, 0·13 %; KH_2PO_4, 0·03 %; K_2HPO_4, 0·10 %; $MgSO_4.7H_2O$, 0·01 %; adjusted to pH 6·9 to 7·2) and both sieve and buffer were kept in motion. The filtrate was poured into a second beaker and the sieve was further washed in fresh buffer several times until the washings were clear. The total filtrate was then put through the second sieve which was similarly washed and finally the process was repeated with the third sieve. Tests showed that movement of the fluid during washing and several washings of each sieve were essential to free the debris of ciliates. Numerous examinations of the residue in the sieve showed that practically all the organisms were washed through when the above method was used. This applied to the rumen contents of sheep on diets of hay, grass or concentrates. When 100 ml. rumen liquor samples were used it was found necessary to put the sample through the first sieve in two 50 ml. quantities. All the sieving was carried out in vessels standing in a 38° water bath.

After sieving, the organisms from the original sample were all present in a large volume of buffer, *c.* 2 l. for a 100 ml. sample. The organisms were then reconcentrated by centrifugation. The volume was first spun at 630 ***g*** for 10 min., the deposits washed out with buffer and then pooled into one centrifuge pot and spun for 5 min. at the same speed. The supernatant fluid was then free of protozoa when the sample was originally from a sheep fed hay and concentrates, but for different diets the speed and time of centrifugation might have to be varied: e.g. the time prolonged slightly for rumen liquor from a grass-fed animal. Excessive speed bursts the holotrichs and must be avoided.

Finally all the protozoa from the original sample were gathered into one centrifuge pot and were washed into the container from which samples were to be taken. This suspension was then made up to the desired volume and diluted with one volume of glycerol. The organisms were left for 15 min. to recover shape. Further dilutions to give a suitable concentration for counting were made from this suspension, using a mixture of 50% (v/v) glycerol in buffer solution.

Sampling of rumen contents and of protozoal suspensions. Important stages in the counting technique were the original removal of a rumen liquor sample from the sheep and the removal of samples to fill the counting chamber. All suspensions were made up in glycerol + buffer mixture in boiling tubes. The protozoa do not cling to the smooth sides of these tubes. A suspension was mixed by inverting the tube eight times while holding a hand over its mouth for the whole period of mixing so that any suspension removed would not affect the final concentration. After this mixing, the 1 ml. samples, to be diluted if necessary, were removed by pipette. The tips of the pipettes were ground down to be 2 mm. in internal diameter; this is necessary to allow free passage of large and small organisms (Adam, 1951). The tip of the pipette was placed centrally in the tube before the sample was removed to decrease to a minimum error due to sedimentation. The viscosity of the suspensions was such that it took 5 min. for starch-filled holotrichs to settle out.

The samples from the rumen were drawn by suction through a glass tube inserted into the fistula. The exact volume to be used in the count was drawn off so that no sampling from rumen contents *in vitro* was required, nor was any further sampling necessary until the glycerol was added. Rumen samples from the bottom or middle of the rumen were obtained by maintaining pressure on the upper end of the tube until it had been inserted into the rumen to the correct level.

RESULTS

Test of cell with washed suspensions of holotrichs

Experiment a. It had been shown by Student (1907) and confirmed by many subsequent workers that, provided no overcrowding occurs, there is a Poisson distribution of particles over the area of a haemocytometer. The similarity in principle of our counting slide to the haemocytometer led us to expect a similar result. To test this two fillings of the slide were made with mixed suspensions of *Isotricha* and *Dasytricha* spp. obtained from the rumen of a sheep fed hay and concentrates. The area of the slide was divided into 209 fields of 1 mm.^{2} by using the vernier scale of the microscope. Each field might contain at least 50 of the Isotrichs or some hundreds of the Dasytrichs and the concentrations of the suspensions were such as to avoid any possibility of overcrowding. The results of this experiment are given in Table 1. Apart from the concentration of *Dasytricha* spp. from suspension A, the means were rather small, and so, following Anscombe (1948), (count + 0·375)$^{\frac{1}{2}}$ was analysed for those three sets of counts. For the fourth, (count)$^{\frac{1}{2}}$ was examined. Table 1 shows that the residual mean square was near the theoretical value of 0·25, and that the variability due to changes along or across the slide was not significantly greater. The data were therefore in accord with a Poisson distribution of the organisms over the slide.

Table 1. *Test of the counting method using two similar washed suspensions of rumen holotrich protozoa*

Two samples of each suspension were counted.

	Degrees of freedom	Suspension A		Suspension B	
		Isotricha spp.	*Dasytricha* spp.	*Isotricha* spp.	*Dasytricha* spp.
Mean count/mm.^{2} field	—	1·0	5·3	1·7	1·4
Residual mean square of transformed counts	180	0·20	0·30	0·27	0·28
Mean square attributable to changes along the slide	18	0·23	0·31	0·37	0·24
Mean square attributable to changes across the slide	10	0·26	0·39	0·41	0·14

It was thus possible to estimate, for a given average size of count, the number of fields which it is necessary to sample in order to estimate the concentration of organisms with some specified accuracy. The results for some representative accuracies are given in Fig. 1. These may be interpreted as follows. Suppose the average concentration is about 20/field, and we wish to estimate the concentration to within 5%. From the 5% curve we can see that if we count 80 fields the true mean will lie within ±5% of the sample mean, *c.* 95 times out of every 100. From the nature of the distribution there will be some asymmetry, but the figure remains a useful guide to the sampling intensity required.

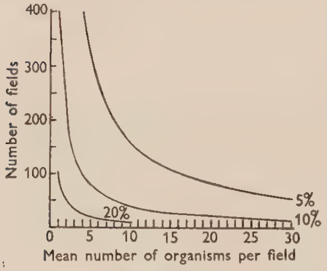

Fig. 1

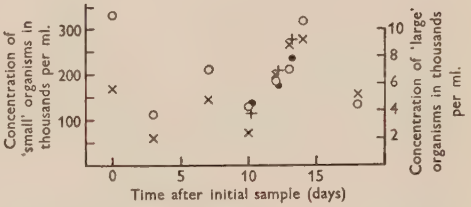

Fig. 2

Fig. 1. The number of fields which it is necessary to count for different concentrations of organisms so that the 95 % confidence limits of the true mean should be within approximately 5 %, 10 %, or 20 % of the sample mean.

Fig. 2. Concentration of 'large' and 'small' organisms in rumen contents of a sheep sampled on different days.

Test of the reliability of the slide counts as representatives of the volume sampled

Experiment b was carried out to see how well the sample drawn from a slide represented the material of its origin. Ideally, one would have a volume of a known concentration of holotrich organisms and sample this several times and examine the counts for bias and variability. It was not practicable to do this, nor was it considered at all likely that any bias would arise, so that this test was restricted to examining the differences which occurred among slides from one suspension. Experiments were carried out which exposed sources of differences between slides, until eventually the technique of mixing described above was evolved. Three experiments were then made to test the sampling method. In each of these washed holotrich organisms were prepared, and suspensions in glycerol + buffer solution were made. A sufficiently large volume was made up to render unimportant the effect of finite sampling fractions (Quenouille, 1950). The slide was filled seven times from each suspension and the organisms counted in thirty fields chosen at random from each slide. Table 2 lists the principal results of these experiments, in which $(\text{count}+0\cdot375)^{\frac{1}{2}}$ was analysed. The listed mean squares of the transformed count confirmed the result of Expt. *a* in showing good agreement with 0·25. For each solution the mean counts on each side agreed well with one another; an analysis of variance of the transformed counts showed that slide differences were not significant. The appropriate mean squares are given in the last two rows of Table 2.

A test of the method applied to total rumen contents

A Cheviot ewe fed a constant daily ration of 450 g. hay and 450 g. concentrates was used throughout Expt. *c*. At 10 a.m. on each of 8 days (over a 19-day period) one 100 ml. sample was withdrawn from the top, and another from the bottom, of the rumen. At 3 p.m. on 3 of these days two 100 ml.

samples were taken from the middle of the rumen. The ciliates were prepared for counting as described above, and as far as possible each stage in the preparation was carried out concurrently for each pair of samples. The organisms from each 100 ml. sample were finally concentrated in 60 ml. of glycerol + buffer solution. To facilitate counting the 'large organisms' (large oligotrichs and *Isotricha* species) a 1/5 dilution was used, and for 'small organisms' (*Dasytricha*, *Diplodinium* and *Entodinium* species) the solution was diluted 1/40. Two slides were prepared of each suspension, and the large organisms were counted in thirty fields on each slide of the more concentrated solution, the small organisms in twenty fields on each of their slides. When the within-slide variability of the transformed count was analysed, the mean squares ranged from 0·16 to 0·38, with an average value of 0·29, so that the protozoa were distributed over each slide in a random manner.

Table 2. *Test of the counting method using three washed suspensions of rumen holotrich protozoa*

The counting chamber was filled seven times from each suspension and the organisms each time counted in 30 fields chosen at random.

Filling of counting chamber	Suspension A		Suspension B		Suspension C	
	Mean count	Mean square of transformed count	Mean count	Mean square of transformed count	Mean count	Mean square of transformed count
1	8·6	0·30	9·8	0·27	11·8	0·29
2	8·2	0·19	9·9	0·18	12·1	0·26
3	10·2	0·30	9·3	0·36	12·1	0·22
4	8·3	0·26	9·2	0·36	11·3	0·16
5	8·7	0·30	9·3	0·32	10·6	0·32
6	8·4	0·29	10·9	0·33	11·6	0·35
7	8·5	0·26	11·4	0·22	11·1	0·19
Mean square attributable to differences between slides (6 D.F.)	0·26		0·47		0·17	
Overall within-slide mean square (203 D.F.)	0·27		0·29		0·26	

Two analyses of the 10 a.m. samples were carried out, one for large and one for small organisms, transforming to the square root of the total count on each slide. For neither analysis were differences significant between slides from the same sample. The mean square attributable to this source of variability was 0·21 based on 14 degrees of freedom for each analysis. Thus, so far as variability over a slide and differences from one slide to another were concerned, the same conclusions appeared to hold for the organisms from rumen contents as for the organisms in washed suspensions.

This experiment tested three additional sources of variability: (1) whether there was a difference between top and bottom of the rumen; (2) whether there were differences from one day to another; (3) whether the difference between top and bottom varied from day to day. The answers obtained were as follows: (1) There was no evidence of a top–bottom difference in concentration of large organisms but the small organisms seemed to be more concentrated at the top. The 8% difference between top and bottom did not quite reach significance at the 5% level. (2) There were large day-to-day differences in the concentration of both large and small organisms ($P < 0{\cdot}001$). When the mean concentrations were plotted against time of sampling, as in Fig. 2, the 3 p.m. samples from the middle of the rumen agreed well with the corresponding 10 a.m. samples, indicating, as expected, that the concentration changed gradually with time. Fig. 2 also revealed a positive day-to-day correlation between the concentrations of large and small organisms. (3) The difference between top and bottom did not vary significantly from day to day.

DISCUSSION

From the results of the experiments described above, it would seem that the MacMaster type of counting chamber used with suspensions of ciliates or samples of rumen contents prepared by the methods described, will enable a count of the organisms to be made with an accuracy which can be determined, and which varies according to the numbers counted. A further implication of the results of Expt. *a* and *b* is that the method developed for sampling and mixing does not appear to introduce any additional source of error. It would thus not be necessary to take a large number of samples from a suspension for counting. For any given concentration the total number of fields counted is the important factor which decides the accuracy of an estimate, and three slides should be ample as a check against an error in filling a slide. From the result of Expt. *c*, namely that the position-by-day interaction was not significant, it seemed likely that repeat samples from a given position in the rumen would not vary more than might be expected on the basis of a random distribution of protozoa in that neighbourhood. In order to examine differences in concentration over the rumen, the top and bottom were chosen, as they seemed most likely to represent the extremes of protozoan concentration, as one might expect a higher concentration of organisms to be associated with the greater quantity of hay fragments nearer the top of the rumen. Although there is danger in generalizing, in our case it appeared that the difference in concentration might not be more than about 10% in favour of the top. Should this be the case generally, then it can be shown that provided no more than 200 organisms/slide are counted, the standard deviation will be only about 10% greater than if the distribution were random over the rumen. It has also been clearly demonstrated that the concentration of organisms changed with time, and that both large and small organisms followed a similar trend. From the magnitude of these changes and from such information as exists in the literature concerning differences between sheep (Williams & Moir, 1951), there is little doubt that variability arising from those two sources would swamp such variability as exists in the sampling methods described in this paper.

The authors wish to express their indebtedness to Dr A. E. Oxford, for much helpful discussion.

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REFERENCES

Adam, K. M. G. (1951). The quantity and distribution of the ciliate protozoa in the large intestine of the horse. *Parasitology*, **41**, 301.

Adam, K. M. G. (1953). *In vivo* observations on the ciliate protozoa inhabiting the large intestine of the horse. *J. gen. Microbiol.* **9**, 376.

Anscombe, F. J. (1948). The transformation of Poisson, binomial and negative-binomial data. *Biometrika*, **35**, 246.

Ferber, K. E. (1928). Die Zahl und Masse der Infusorien im Pansen und ihre Bedeutung für den Eiweissaufbau der Wiederkäuer. *Z. Tierz. ZüchtBiol.* **12**, 31.

Ferber, K. E. & Winogradawa-Federowa, T. (1929). Zählung und Teilungsquote der Infusorien im Pansen der Wiederkäuer. *Biol. Zbl.* **49** (6), 321.

Gilbert, J. Y. (1942). The errors of the Sedgwick-Rafter counting chamber in the enumeration of Phytoplankton. *Trans. Amer. micr. Soc.* **61**, 217.

Gutierrez, J. (1955). Experiments on the culture and physiology of holotrichs from the bovine rumen. *Biochem. J.* **60**, 516.

Hall, R. P., Johnson, D. F. & Loefer, J. B. (1935). A method for counting protozoa in the measurement of growth under experimental conditions. *Trans. Amer. micr. Soc.* **54**, 298.

Heald, P. J., Oxford, A. E. & Sugden, B. (1952). A convenient method for preparing massive suspensions of virtually bacteria-free ciliate protozoa of the genera *Isotricha* and *Dasytricha* for monometric studies. *Nature, Lond.* **169**, 1055.

Hungate, R. E. (1942). The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. *Biol. Bull., Woods Hole*, **83**, 303.

Johnson, B. C., Hamilton, R. S., Robinson, W. R. & Garey, J. C. (1944). On the mechanism of non-protein-nitrogen utilization by ruminants. *J. Anim. Sci.* **3**, 287.

Mangold, E. (1929). *Handbuch der Ernährung und des Stoffwechsels der Landwirtschaftlichen Nutztiere*, Band II. Berlin: Julius Springer.

Moir, R. J. (1951). The seasonal variation in the ruminal micro-organisms of grazing sheep. *Aust. J. agric. Res.* **2**, 322.

Moir, R. J. & Somers, M. (1956). A factor influencing the protozoal population in Sheep. *Nature, Lond.* **178**, 1472.

Mowry, H. A. & Becker, E. R. (1930). Experiments on the biology of infusoria inhabiting the rumen of goats. *Iowa St. Coll. J. Sci.* **5**, 35.

Oxford, A. E. (1955). The rumen protozoa: their chemical composition, metabolism, requirements for maintenance and culture and physiological significance for the host. *Exp. Parasitol.* **4**, 569.

Quenouille, M. H. (1950). *Introductory Statistics.* London: Butterworth-Springer.

'Student' (1907). On the error of counting with a haemocytometer. *Biometrika*, **5**, 351.

Wath, J. G. van der & Myburgh, S. (1941). Studies on the alimentary tract of merino sheep in South Africa. VI. The role of infusoria in ruminant digestion with some remarks on ruminant bacteria. *Onderstepoort J. vet. Sci.* **17**, 61.

Weller, R. A. & Gray, F. V. (1954). The passage of starch through the stomach of the sheep. *J. exp. Biol.* **31**, 40.

Williams, V. J. & Moir, R. J. (1951). Ruminal Flora Studies in the sheep. III. The influence of different sources of nitrogen upon nitrogen retention and upon the total number of free micro-organisms in the rumen. *Aust. J. sci. Res.* **4b**, 377.

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The Occurrence of Rare Motile Bacteria in some Non-motile Salmonella Strains

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SUMMARY: The presence of rare motile bacteria in 22 of 48 non-motile Salmonella strains tested has been inferred from the production of satellite micro-colonies in semi-solid medium. Such rare motile bacteria have been isolated by micromanipulation; they are not motile mutants, since their progeny are nearly all non-motile. They transmitted motility to only a few of their descendants, as if their motility were due to the presence of non-multiplying motility-conferring particles which were transmitted to their progeny at cell division. It is inferred that small numbers of such particles arise during an intracellular 'event'; this event probably consists of a transient ability to synthesize new flagella. The frequency of events in one Salmonella O strain was found to be *c.* 4×10^{-5}/bacterium/generation.

In many bacterial genera both motile and non-motile strains are found; for instance in *Salmonella* (see Stocker, Zinder & Lederberg, 1953). Strains in which a small minority of cells are motile have been reported, e.g. Jordan, Caldwell & Reiter (1934); such strains are usually termed 'poorly motile'. The possible significance of 'poor motility' as an indication of cellular variation has received little attention. Stocker *et al.* (1953), in the course of experiments on the transduction of motility, grew Salmonella strains on a semi-solid medium; motile strains gave spreading growth, whereas completely non-motile strains grew only at the site of inoculation. They noted, however, that in some 'non-motile' strains the growth at the site of inoculation was surrounded by satellite micro-colonies, which on subculture yielded only non-motile growth indistinguishable from the original strain; antiflagellar serum prevented the formation of the satellite colonies. Stocker *et al.* suggested that in the strains concerned a very small fraction of the cells were motile and migrated through the agar, but produced non-motile progeny, constituting the satellite micro-colonies.

In subsequent experiments on the abortive transduction of motility Stocker (1956*b*) and Lederberg (1956), by micromanipulation, isolated motile bacteria from suspensions of non-motile Salmonella strains which had been treated with phage lysates of motile strains (i.e. lysates capable of transducing motility); in these experiments they encountered a class of motile cells which transmitted motility down only one line of descent to a single one of their progeny of each generation. Stocker (1956*b*) supposed that this *unilinear transmission* of motility (discussed by Stocker, 1956*a*) resulted from the transmission of a hypothetical non-replicated cell structure, the motility-conferring (MC)

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particle, which was perhaps a flagellum (or its basal granule) or a cell wall bearing one or more flagella. Stocker (1956*b*) concluded that in abortive transduction of motility the synthesis of motility-conferring particles by a small minority of the bacteria resulted from the presence in each such bacterium of a transduced gene (which also was transmitted unilinearly).

The synthesis of motility-conferring particles by a small proportion of bacteria in a culture, resulting from causes other than transduction, is inferred from the observations described in the present paper. The spontaneous production of satellite colonies has been investigated in a considerable number of non-motile strains of *Salmonella* and other genera, and the spontaneous occurrence of rare motile bacteria in some satellite-producing non-motile Salmonella strains has been directly demonstrated. Such bacteria have been isolated by micromanipulation and shown to produce mainly non-motile progeny with only a few motile descendants, each of which transmits motility unilinearly. It is suggested that these motile bacteria, which are demonstrably not true motile mutants, result from 'events' consisting of the transient ability of a cell to synthesize particulate locomotor apparatus (MC particles). A method of measuring the frequency of such events per bacterium per generation has been devised.

METHODS

Organisms. Strains used in this work are listed by Quadling (1956*a*). Non-motile Salmonella strains were principally obtained by courtesy of Prof. J. Lederberg, Department of Genetics, University of Wisconsin, Wis., U.S.A., and of Dr Joan Taylor, at the Salmonella Reference Laboratory of the Public Health Laboratory Service, Colindale, London, N.W. 9. Many of these strains are listed by Stocker *et al.* (1953). *Vibrio cholerae* O strains were obtained from Dr K. Bhaskaran at the Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2. Proteus O strains were obtained from the National Collection of Type Cultures. *Escherichia coli* O strains of serotypes O111 and O55 were provided by Dr Joan Taylor.

Media. Nutrient broths were made from a tryptic digest of lean beef or from a papain digest of horse meat (Asheshov, 1941) and nutrient agar by addition of 2% (w/v) Japanese agar. Semi-solid or 'motility' medium was that of Edwards & Bruner (1942) as used by Stocker *et al.* (1953). It contains 0·4% (w/v) 'Difco' agar and 8·0% (w/v) 'Difco' gelatine in a peptone broth basal medium. In some experiments only 4% gelatine was used and in others gelatine was omitted altogether. Such omissions are mentioned in the description of experiments.

Stock cultures. Cultures were maintained on Dorset's Egg slopes, which were generally kept at laboratory temperature. In some cases stock cultures were started from a single bacterium isolated by micromanipulation. Such single-cell cultures were stored at 4°; they are designated by the appropriate strain number, followed by an oblique stroke and a capital letter, e.g. SL191/G indicates a culture derived from a single bacterium, picked by micromanipulation, from a culture of strain SL191.

Cultures used in experiments. All cultures were grown at 37°, unless otherwise stated. A streak from the stock culture was plated-out, so as to give discrete colonies after overnight incubation; a colony was then subcultured into nutrient broth. All experiments were carried out with bacteria which were in the logarithmic phase of growth. Cultures were grown unshaken in loosely capped tubes, or 20 ml. McCartney bottles. Viable counts were made by the method of Miles & Misra (1938), or by spreading samples of 0·1–0·5 ml. of suitable dilutions over the surface of nutrient agar plates.

Motility. Broth cultures were examined for motility by low-power dark-ground microscopy, as described by Stocker (1956*b*). The motility characteristics of cultures were also inferred from the colony patterns produced during growth on semi-solid medium (Pl. 1, figs. 1, 2).

Micromanipulation. The methods used were those of de Fonbrune (1949) as described by Stocker (1956*b*).

RESULTS

Behaviour of non-motile strains grown in semi-solid medium

A pour-plate technique was used to test non-motile strains for the production of satellite micro-colonies. Cultures in logarithmic growth in broth at 37° are suitably diluted. Melted semi-solid medium (0·3 % (w/v) agar, no gelatine) at 44° is then inoculated with the diluted culture so as to give about 2 bacteria/ml. of medium. This inoculated medium is at once poured into 10 cm. Petri dishes and incubated at 37°. After 16–18 hr. incubation, colonies derived from the individual organisms of the inoculum can be distinguished. Positive results are indicated by the presence of satellite micro-colonies associated with the larger parent colonies. All colonies are well defined and most of the satellites are clearly separated from the parent colonies (Pl. 1, fig. 3).

Forty-eight non-motile Salmonella strains (of species other than *S. gallinarum* and *S. pullorum*) were tested in this way and satellites found with 22 of them. Eight of the strains tested were flagellated and non-motile ('paralysed') strains. Three of the paralysed strains produced satellites. These results are summarized in Table 1, which also records results of tests with some non-motile strains of genera other than Salmonella.

Table 1. *Results of tests of non-motile strains in semi-solid medium. Production of satellite micro-colonies indicates the presence of rare motile bacteria*

	Satellite micro-colonies		
Non-motile strains	present	absent	Total
Salmonella O strains*	19	21	40
Salmonella flagellated but non-motile strains*	3	5	8
Escherichia coli O strains	3	4	7
Proteus O strains	1	2	3
Vibrio cholerae O strains	5	0	5
Totals	31	32	63

* Salmonella strains of species other than *S. gallinarum* and *S. pullorum*.

The numbers of satellites produced during overnight incubation at 37° varied from strain to strain. In some strains an average of only one or a few satellites were present per colony; in others there were many satellites round each colony. Within a given strain the frequency of satellite production remained approximately constant from experiment to experiment, except that appearances varied with different batches of medium; in general the stiffer the medium the fewer the satellites. The medium used here contained no gelatine in order to make the tests as sensitive as possible.

The production of satellites by some non-motile Proteus, Vibrio and Escherichia strains shows that this phenomenon is not confined to a single group of organisms. Satellite formation was also looked for in species and genera characterized by non-motility: no positive results were obtained in tests of about forty Shigella strains or of about thirty *Salmonella gallinarum* and *S. pullorum* strains.

Estimation of satellite frequency

Quantitative data on satellite frequency were obtained by an elaboration of the technique described above. Estimates were made of the rate at which satellites (and by inference, motile individuals) were produced during growth of a non-motile *Salmonella typhimurium* O strain SW 545 (Stocker *et al.* 1953). In the experiments reported here a streptomycin-resistant mutant (SL 191/G) of this strain was used.

Melted semi-solid medium (0·4%, w/v, agar, 4%, w/v, gelatine) was inoculated with a culture of SL 191/G which had previously been in logarithmic growth at 37°. It was arranged that about 2–5 bacteria were present in each ml. of semi-solid medium. The inoculated medium was at once poured into pre-warmed 10 cm. Petri dishes in 20 ml. amounts which were immediately incubated at 37°. It was assumed that each viable bacterium would grow and divide to form a colony; motile bacteria arising during growth of these colonies would swim out into the medium and initiate satellite micro-colonies.

After 5 hr. at 37° the plates were chilled, to set the medium, and were then left at bench temperature (*c.* 20°). Because the medium contained gelatine it was solid at bench temperature; no further bacterial movement could occur. However, growth continued at this temperature until both parent and satellite colonies were visible. After some 18 hr. at 20° the plates were examined under a magnification of *c.* × 10 and the proportion of parent colonies with and without satellites was determined.

The average number of colonies/plate was calculated from viable counts made on the inoculum. The number of bacteria/plate after 5 hr. at 37°, i.e. at the time of chilling, was determined from a replicate plate which itself was not chilled. The entire contents of this plate were suspended, by means of a magnetic stirrer, in a known volume of broth and a viable count made. The ratio of inoculum number to final bacterial number gives a value for the average number of bacteria per colony at the conclusion of incubation at 37°.

We believe that satellite micro-colonies arise as a result of intra-bacterial 'events' whose nature will be discussed later. The frequency of such satellite-producing events/bacterium/generation may be calculated from the data on the following assumptions: (1) that the probability per bacterium of suffering an event does not vary from one colony to another or at different times during the growth of a colony, and (2) that there is no great variation in population size between colonies in a single plate. The data to be described support the first assumption; counts of viable bacteria in colonies picked individually, under the microscope, showed that the second assumption was reasonable. On these assumptions the proportion of colonies with *no* satellites (P_0) can be put equal to e^{-z}, the first term of the Poisson summation. In this expression z is the average number of events per colony. From the average number of events calculated in this way, and the average number of bacteria per colony, the event frequency per bacterium per generation can be calculated by the use of a suitable formula, e.g. formula (1) of Newcombe (1948).

The results of two experiments of this type are summarized in Tables 2 and 3. An event frequency of c. 4×10^{-5} per bacterium per generation was found for SL191/G. The observed average number of satellites per colony exceeded, by a factor of between two and three, the average number of events per colony calculated from the Poisson distribution. If the hypothesis of satellite-producing events occurring at random is correct this means that an event results in the appearance of an average of several satellites.

Table 2. *Determination of event frequency per bacterium per generation by pour-plate method: strain SL*191/*G incubated for* 5 *hr. at* 37° *in semi-solid medium; colonies examined for satellites after further growth at* 20°

Expt. no.	Colonies examined for satellites: Negative	Colonies examined for satellites: Total	Proportion negative (P_0)	Calculated mean number of events per colony*	Calculated mean number organisms per colony†	Calculated event frequency‡
I	164	465	0·353	1·04	$1{\cdot}33 \times 10^4$	$5{\cdot}5 \times 10^{-5}$
II	100	263	0·38	0·97	$3{\cdot}07 \times 10^4$	$2{\cdot}2 \times 10^{-5}$

* Calculated from the first term of the Poisson series, $P_0 = e^{-z}$, where z is the mean number of events per colony.

† Calculated from viable counts at beginning and end of incubation at 37°.

‡ Per bacterium per generation.

Table 3. *Distribution of numbers of satellites per colony in Expt.* 1 *of Table* 2

No. of satellites	0	1	2	3	4	5	6	7	8	9	10	11
Frequency	30	14	8	8	7	8	0	3	3	1	2	1

Mean number of satellites/colony	2·44
Calculated mean number of events/colony	1·04
Mean number of satellites/event	$2{\cdot}44/1{\cdot}04 = 2{\cdot}34$

Estimates of event frequency were also made at different times during the growth of the parent colonies by withdrawing replicate plates from incubation at intervals between 4 and 6 hr. (Table 4). As would be expected if events occur with a constant probability per bacterium per generation, the mean number of satellites per colony and the calculated mean number of events per colony increased with increasing duration of incubation at 37°, i.e. with increasing number of viable organisms per colony: but there was little divergence between estimates of numbers of satellites per event made at different times. This indicates that the assumed Poissonian distribution of numbers of *events* per colony is probably justified. If, as it now appears, several satellites result from one event, then the distribution of numbers of *satellites* per colony will not be Poissonian. It is evident from the data of Table 3 that the distribution of numbers of satellites per colony is non-Poissonian.

Table 4. *Numbers of satellites per colony, in pour-plates, after various times of incubation at 37°*

Pour-plates of strain SL191/G were withdrawn from incubation at 37° at stated times, chilled, and examined for satellites after further growth at 20°.

Time at 37° (hr.)	Colonies examined	Proportion negative	Calculated mean number of events/colony	Average number satellites/colony	Mean number satellites/event
4	131	0·72	0·33	0·78	2·35
4½	148	0·66	0·42	0·91	2·15
5	143	0·42	0·87	1·8	2·07
5½	152	0·28	1·26	2·87	2·27
6	151	0·1	2·31	5·89	2·55

The estimates of event frequency are minimum estimates and no high degree of precision can be claimed for them. Their accuracy depends largely on the efficiency of detection of motile bacteria by the pour-plate method. As mentioned above, the stiffer the medium the fewer the satellites found; small variations in stiffness occur from experiment to experiment and even from plate to plate within a single experiment. It is possible that a class of motile bacteria unable to swim in this medium remained undetected. Furthermore it is likely *a priori* that there will be some delay between the event and the initiation of the first resulting satellite, for a bacterium in which an event occurs will not become motile until it has grown functional flagella. Consequently those events which have only just occurred at the time of chilling the plates will not be detected and the calculated event-frequency will be an underestimate. However, similar values for event frequency were obtained in similar experiments carried out on different occasions.

Isolation of motile bacteria by micromanipulation

Stocker *et al.* (1953) suggested that a small fraction of the bacteria might be motile in O strains which produced satellites during growth on semi-solid medium. This suggestion was verified by experiments in which growing, standing-drop, cultures of SL191/G were observed by low-power dark-ground microscopy. Each drop was *c.* 10^{-3} ml.; evaporation was minimized and contamination avoided by the use of a thin protective film of mineral oil. As expected, motile bacteria were present in very small numbers; they were rarely detectable before about 10^6 bacteria were present in a drop, when on average about ten motile bacteria were seen.

Subculture of satellite micro-colonies yielded non-motile growth with the characteristics of the parent O strain, which suggested that motility of the satellite-initiating bacteria was transitory. It was therefore of interest to isolate such motile bacteria by micromanipulation and to observe their behaviour.

Large populations of strain SL191/G, in exponential growth, were examined in hanging drop, using the oil-chamber technique described by Stocker (1956*b*). Rare motile bacteria were seen and a number were transferred, each to a separate micro-drop of sterile broth. The isolated motile bacteria were allowed to grow and divide at *c.* 20°, and the resulting populations were examined when between 200 and 300 bacteria were present in each drop.

The progeny of these motile bacteria were, for the most part, non-motile. However, in most populations a few motile bacteria were found amongst the non-motile majority (Table 5). Had the original motile bacteria been motile mutants one would have expected them to give rise to motile populations. The results obtained suggested that the original motile bacteria were able to transmit motility to few, if any, of their progeny.

Table 5. *Populations derived from individual motile bacteria, isolated from a culture of strain SL*191/*G by micromanipulation, examined after about eight generations growth at* 20°

Description of population	Frequency (populations)
All bacteria non-motile	17
1 motile bacterium, rest non-motile	27
2 motile bacteria, rest non-motile	6
3 motile bacteria, rest non-motile	2
4 motile bacteria, rest non-motile	1
Total no. of populations	53

A similar situation was encountered by Stocker (1956*b*) and by Lederberg (1956) during investigations on the transduction of motility to non-motile Salmonella strains. To account for his results, Stocker put forward the hypothesis of unilinear transmission of motility resulting from the unilinear transmission of motility-conferring (MC) particles, referred to in the introduction to this paper. On this hypothesis a bacterium with only one MC particle will give rise to a clone containing only one motile descendant. Repeated isolation of the motile bacterium, at intervals during growth of the clone, would constitute a critical test of the hypothesis: if the hypothesis is correct the re-isolated motile bacterium should always produce one motile descendant and its non-motile progeny should produce only non-motile descendants. An experiment of this sort will be described next.

A motile bacterium was isolated from a culture of SL191/G and grown in a micro-drop of broth. This micro-culture and the others arising from it in the course of the experiment were inspected at intervals. Whenever a motile bacterium was observed it was removed to a fresh drop of broth. The course of this experiment is summarized in Fig. 1. During its first four divisions the isolated motile bacterium gave rise to two motile and a number of non-motile progeny and at no time during the experiment were more than two motile bacteria found amongst the descendants of the original motile isolate. The transmission of motility was followed for respectively *c.* eleven and *c.* sixteen generations in each of the two lines; thereafter no motile descendants were seen.

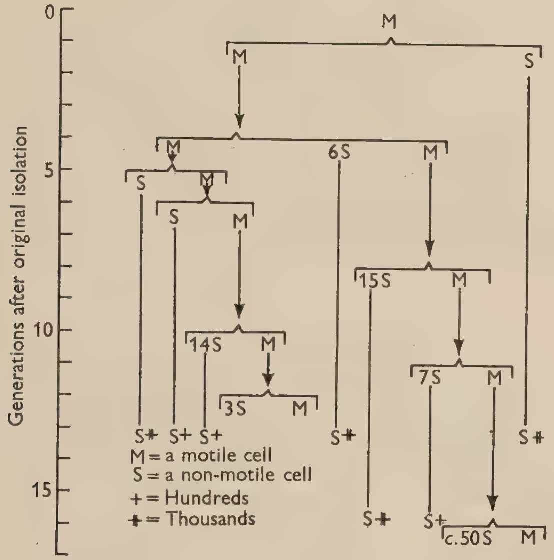

Fig. 1. Behaviour of the progeny of a motile bacterium isolated by micromanipulation from a culture of *Salmonella typhimurium* O strain SL 191/G. Micro-cultures were incubated at room temperature (*c.* 20°) and examined at intervals; motile bacteria were re-isolated to separate droplets as indicated by arrows.

In this series of experiments about twenty motile bacteria were tested by repeated reisolation in this way. The shortest observed 'life' of an assumed MC particle was one generation, i.e. at the two-cell stage neither daughter of a motile bacterium was itself motile. The most persistent transmission of motility lasted *c.* 16 generations; persistence for *c.* 5 to 10 generations was typical. All the motile cells behaved in a manner consistent with the MC particle hypothesis. Additional evidence in favour of the hypothesis was provided by observations on the fission of bacteria inferred to have one MC particle. Such bacteria were occasionally observed at the moment of fission: one daughter remained motile whilst the other was non-motile from the instant of separation.

No motile bacteria were seen in about 30% of the micro-cultures described in Table 5. Assuming the hypothesis of MC particles, there are several possible explanations for this. Motile bacteria can become mechanically immobilized by sticking at an interface of the hanging drop and this was often observed; the particle-bearing cell might die, the particle itself might cease its activity, and the observer might miss a motile bacterium amongst many non-motile bacteria. The numbers of motile organisms recorded must be regarded as minimum estimates. Possible observer bias, e.g. in length of time devoted to examination of a particular micro-culture, was reduced by working concurrently with a number of unrelated micro-cultures—the course of experiments was reconstructed in 'pedigree' form only when work had ceased. If more than two motile bacteria had been present in the lineage illustrated (Fig. 1) they should have been detected at some stage during the experiment. The repeated detection of such motile bacteria over a number of generations of fission suggests that the efficiency of detection is high. The results in Table 5 bear on this point, for the micro-cultures in which no motile bacteria were detected arose *ex hypothesi* from bacteria with at least one MC particle. The proportion of such cultures can be regarded as an indication of the probability of failure, from all causes, to detect motile bacteria containing one MC particle during a single period of observation.

DISCUSSION

The presence of rare motile bacteria in a *Salmonella typhimurium* O strain, SL191/G, has thus been investigated in semi-solid medium and by micromanipulation. The evidence obtained from these two lines of work is complementary. The distribution of numbers of satellite micro-colonies (each presumed to arise from a motile bacterium) indicates that 'events' occur, each event giving rise to an average of about 2·5 micro-colonies. Presumably such events give rise to an average of this number of motile bacteria able to swim in the semi-solid medium. Micromanipulation experiments on single motile bacteria provides evidence which supports the hypothesis of MC particles. Taken together, these lines of evidence suggest that the events detected in semi-solid medium result from the synthesis of a small number of MC particles in a hitherto non-motile bacterium.

The suggestion that MC particles are synthesized in 'bursts' of small numbers, presumably as a result of some discontinuous biochemical process, will be referred to as the 'event hypothesis'. It is illustrated in Fig. 2 which represents the occurrence of an event in a non-motile bacterium. An arbitrary duration of less than one generation-time for the event is shown; during this period a small number of MC particles are synthesized. The particle-bearing bacteria become motile and in semi-solid media would initiate satellite micro-colonies. The sharing-out of MC particles amongst the progeny of the cell in which they were synthesized is also illustrated in Fig. 2. Bacteria, inferred in retrospect to have had several MC particles, when isolated gave rise to small numbers of motile progeny during their first few divisions after isolation; thereafter no increase occurred in the numbers of their motile descendants (Stocker, 1956*b*); similar results were obtained in the present work. This implies that MC particles are shared between daughter cells so that eventually each motile bacterium will have only one particle and then no further increase will occur in numbers of motile progeny. If this is so, a bacterium which had several particles when isolated must have been closely related to, or identical with, a bacterium in which an event had occurred.

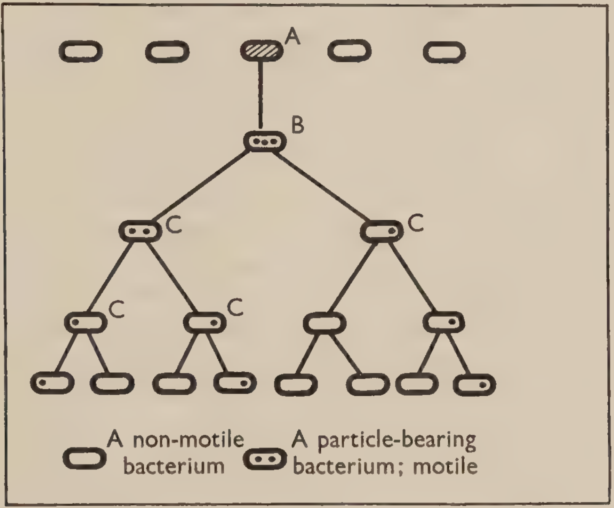

Fig. 2. The event hypothesis: the occurrence of an event (A) in a non-motile bacterium results in (B) the synthesis of a small number of MC particles which are shared-out (C) during growth and division.

The MC particle hypothesis has been fully discussed by Stocker (1956*b*) and by Lederberg (1956). The present work demonstrates that such particles occur spontaneously in the strain SL191/G: they have also been demonstrated by micromanipulation in rare, spontaneously occurring, motile bacteria isolated from two other Salmonella O strains (Quadling, unpublished). Presumably MC particles also occur in all those non-motile strains which produce satellite micro-colonies in semi-solid medium. The evidence obtained in the present studies, though indirect, is consistent with the suggestion that one MC particle corresponds to one flagellum (or, in the case of paralysed strains, to one functioning flagellum). For example, one might expect a bacterium with several flagella (MC particles) to be more strongly motile than a bacterium with only one. If bacteria with one MC particle were able to swim in semi-solid medium then an event occurring early in the growth of a parent colony would be expected to yield many more satellite micro-colonies than a similar event occurring later. That this is not so can be inferred from the fact that the average number of satellites produced per event does not change appreciably during growth of the parent colonies (Table 4). Presumably satellite micro-colonies are initiated by bacteria with several MC particles—during growth and early divisions of such bacteria the number of MC particles per bacterium will be reduced to a critical value below which no swimming can occur in the semi-solid medium. Stocker (1956*b*) also concluded, from experiments on the abortive transduction of motility, that bacteria with several MC particles were able to travel through a semi-solid medium whereas bacteria with only a single particle could not do so. In studies on Salmonella strains in which motile bacteria can occur at high frequency we have shown (Quadling, to be published; Quadling, 1956*a*, *b*) that there is a good correlation between the distributions of numbers of MC particles per bacterium, inferred by micromanipulation studies, and the distributions of numbers of stained flagella per bacterium. We have also shown (Quadling & Stocker, 1956; Quadling, 1956*a*) that flagella are distributed about equally between daughter cells, as was predicted on the basis of the behaviour of MC particles.

The work reported here has shown that in each of a number of Salmonella O strains there is heterogeneity *apropos* of motility. Unless events are so rare as to be undetectable, or so frequent as to occur in all or nearly all individuals, it is clear that there will be detectable heterogeneity in a population. The precise mechanism of an event remains a matter for speculation. In formal, and doubtless oversimplified terms, an affected cell can be regarded as changing from a 'non-synthesizing' state, in which MC particles are not formed, to a transient 'synthesizing' state in which they are. The observations can be formally described in terms of an appropriate random mutation rate to the 'synthesizing' state coupled with a very high back-mutation rate to 'non-synthesizing'. Delbrück (1945) has calculated that a gene mutation with a reversion rate of 0·5 or more per cell generation would be unable to persist to give a mutant clone; a similar back-mutation rate would be required to explain the impersistence of the 'synthesizing' state. It seems implausible to attribute the 'non-synthesizing'$\rightleftharpoons$'synthesizing' transition to gene mutation and almost instantaneous back-mutation; this would predicate a high degree of instability of the genetic material. Whilst such instability cannot be excluded it seems preferable, for economy of hypothesis, to regard these transitions as changes in phenotype only, whose precise cause remains unknown. In the strain studied events leading to satellite formation occur at a rate of about 10^{-5} per bacterium per generation-time; we infer that each bacterium has this probability of undergoing a transient change of phenotype to the flagella-synthesizing state. Such spontaneous transient changes of phenotype with respect to an all-or-none, or discontinuously variable, character may be termed 'phenotypic excursions'. An excursion perhaps results from some underlying continuously variable cell character temporarily passing beyond a threshold value. The postulated phenotypic excursions may perhaps be formally related to quasi-continuous variation in mice (Grüneberg, 1952). Grüneberg's studies on skeletal defects, which appeared in some animals but not others of pure-line strains of mice, led him to suggest that the underlying basis for the defects was a continuous variable associated with a physiological threshold. Possibly quasi-continuous variation results from a physiological 'phenotypic excursion' occurring at a critical stage in development.

The frequency of satellite-producing events, which we interpret as a measure of the probability of phenotypic excursion to the flagella-synthesizing condition, per bacterium per generation time, was approximately constant (in a standard environment) in the strain investigated; in other strains it has been shown to alter through mutation (Quadling, 1956*a*); we conclude that this frequency or probability is genetically determined. Stocker *et al.* (1953) found by transduction experiments that there were a number of genetic loci in Salmonella, mutation at any of which rendered the strain non-motile by preventing the synthesis of flagella; further experiments of the same sort (Quadling, 1956*a*; Stocker & Quadling, unpublished) showed that there are at least thirteen such loci, concerned with the synthesis of functional (i.e. non-paralysed) flagella. The non-motile strains tested in semi-solid medium (Table 1) included thirteen strains, each non-motile through mutation at a different locus; eight of these strains produced satellites. It now appears that the effect of many mutant genes conferring 'non-motility' is to reduce the proportion of bacteria capable of synthesizing effective flagella to a very low level, but not to zero.

In strain SL191/G the great majority of the population are non-motile and flagella cannot be detected in stained preparations, whilst a small minority of the population produce MC particles which we believe to be flagella; we infer that effective synthesis of flagella is confined to a small proportion of the population. This infrequent synthesis of flagella may be formally analogous to 'leakage' described in *Neurospora* spp. by Bonner, Yanofsky & Partridge (1952). They showed that certain mutants which lacked the 'wild-type' ability to synthesize certain growth-factors were able to synthesize very small amounts of the required factor. This biochemical ability they attributed to 'leakage', implying that the genetic block in synthesis was not absolute. In the absence of a character, such as motility, which permits the detection of differences between individuals, the distribution of a given biochemical activity amongst a population remains unknown. Whilst the activity of a population must be the sum of the activities of its component individuals, the present work provides evidence that the activity of the individual is not necessarily the average activity for the population.

We wish to thank those who have provided us with non-motile cultures for use in these studies. Our thanks are due to Dr C. J. Perret and other colleagues and friends for much helpful discussion. It is also a pleasure to acknowledge the technical assistance of Mrs M. Worth. This paper is based on a Ph.D. thesis submitted by one of us (C.Q.) to the University of London, 1956.

REFERENCES

ASHESHOV, I. N. (1941). Papain digest media and standardisation of media in general. *Canad. publ. Hlth J.* **32**, 468.

BONNER, D. M., YANOFSKY, C. & PARTRIDGE, C. W. H. (1952). Incomplete genetic blocks in biochemical mutants of *Neurospora*. *Proc. nat. Acad. Sci., Wash.*, 38, 25.

DELBRÜCK, M. (1945). Spontaneous mutations of bacteria. *Ann. Mo. bot. Gdn*, **32**, 223.

EDWARDS, P. R. & BRUNER, D. W. (1942). Serological identification of *Salmonella* cultures. *Circ. Ky agric. Exp. Sta.* no. 54.

FONBRUNE, P. DE (1949). *Technique de micromanipulation.* Paris: Masson.

Grüneberg, H. (1952). Genetical studies on the skeleton of the mouse. IV. Quasi-continuous variations. *J. Genet.* **51**, 95.

Jordan, E. O., Caldwell, M. E. & Reiter, D. (1934). Bacterial motility. *J. Bact.* **27**, 165.

Lederberg, J. (1956). Linear inheritance in transductional clones. *Genetics*, **41**, 845.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Newcombe, H. B. (1948). Delayed phenotypic expression of spontaneous mutations in *Escherichia coli*. *Genetics*, **33**, 447.

Quadling, C. (1956*a*). *Genetic and environmental determination of bacterial motility, with special reference to Salmonella. Ph.D. thesis.* University of London.

Quadling, C. (1956*b*). In *The Lister Institute of Preventive Medicine: Report of the Governing Body*, 1956, p. 8.

Quadling, C. & Stocker, B. A. D. (1956). An environmentally induced transition from the flagellated to the non-flagellated state in Salmonella: the fate of parental flagella at cell division (Abstract). *J. gen. Microbiol.* **15**, i.

Stocker, B. A. D. (1956*a*). Bacterial flagella; morphology, constitution and inheritance. In *Bacterial Anatomy*, *Symp. Soc. gen. Microbiol.* **6**, 19.

Stocker, B. A. D. (1956*b*). Abortive transduction of motility in Salmonella; a non-replicated gene transmitted through many generations to a single descendant. *J. gen. Microbiol.* **15**, 575.

Stocker, B. A. D., Zinder, N. D. & Lederberg, J. (1953). Transduction of flagellar characters in Salmonella. *J. gen. Microbiol.* **9**, 410.

EXPLANATION OF PLATE

Fig. 1. Appearance of *Salmonella typhimurium* O strain SL191/G on semi-solid medium after 18 hr. at 37°. Numerous satellite micro-colonies are associated with the streaks of bacterial growth present at the sites of inoculation. × 0·9.

Fig. 2. View of the edge of streak shown in Fig. 1. × 20.

Fig. 3. 'Parent' colonies, some of which have associated satellites, from a pour-plate experiment with strain SL191/G in semi-solid medium: 5 hr. at 37°, then 24 hr. at 20°. × 3.

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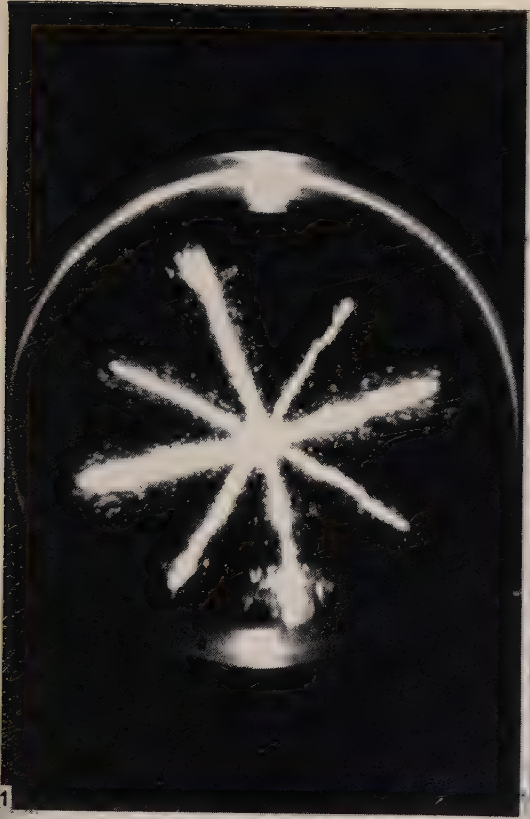

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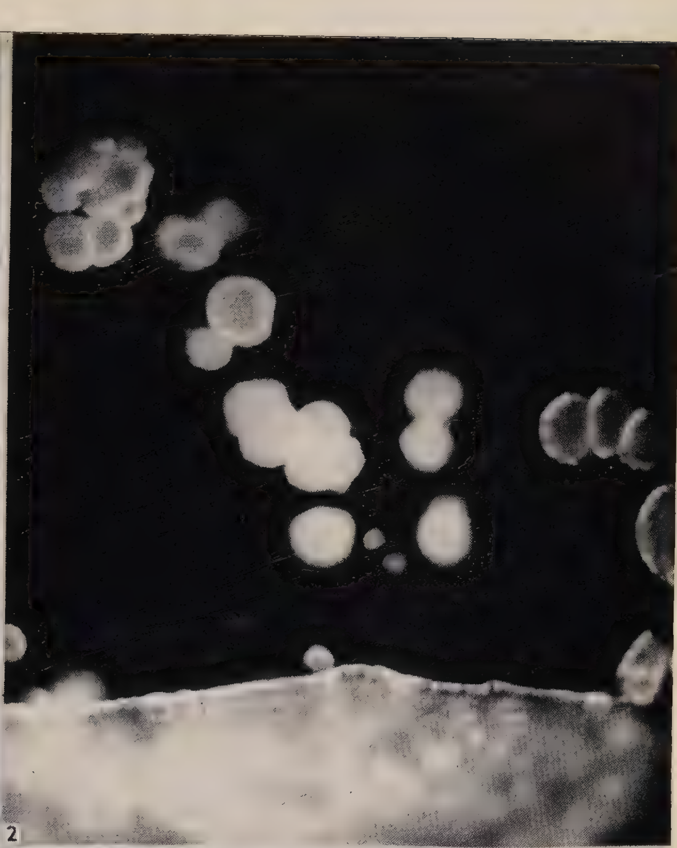

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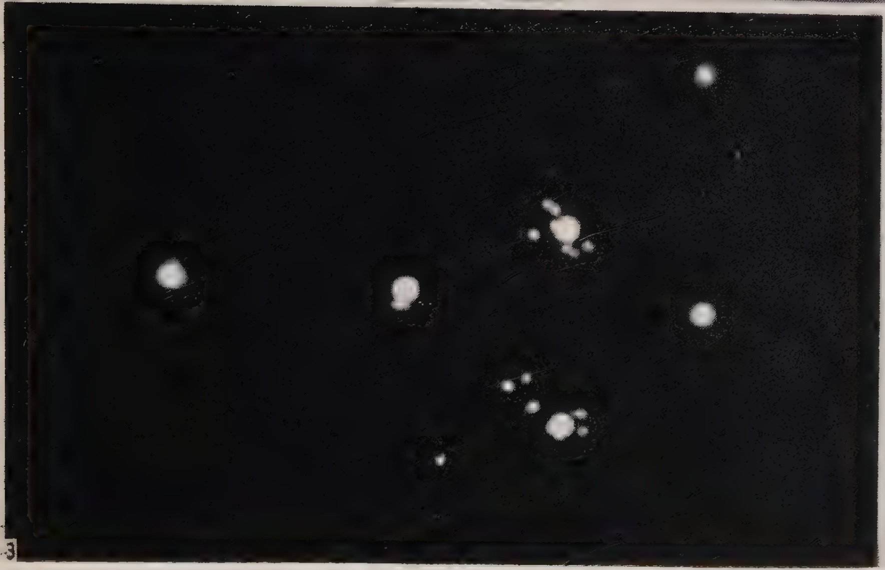

3

C. Quadling & B. A. D. Stocker—Motile bacteria in non-motile strains.
Plate 1

(*Facing p.* 436)

Smith, E. E. B., Mills, G. T., Harper, E. M. & Galloway, B. (1957). *J. gen. Microbiol.* **17**, 437–444

The Cellular Polysaccharide of a Type II non-capsulated Pneumococcus

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SUMMARY: A purified cellular polysaccharide was obtained from a non-capsulated pneumococcus (*Streptococcus pneumoniae*, strain R19, derived from a Type II organism). The main purification steps were peptic, tryptic and ribonuclease digestions, removal of protein by the Sevag technique and ethanol precipitation of the polysaccharide. Ribonuclease digestion was an essential step in the purification procedure. The constituent sugars of the isolated polysaccharide were galactose, mannose and glucosamine. The acetyl content of the polysaccharide indicated that the glucosamine was fully acetylated on the N position and that some *O*-acetyl was also present. The phosphorus content was not entirely explicable in terms of the known ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contamination. Residual amino acid was consistently present which may have been due to bound amino acids or to protein contamination.

A study of the uridine pyrophosphoglycosyl compounds of the pneumococcus (Smith, Mills & Harper, 1957*a*) showed the presence of uridine pyrophosphoglucose (UPPglucose) and uridine pyrophosphoglucuronic acid (UPPglucuronic acid) in *Streptococcus pneumoniae*, strain R19, derived from a Type II organism, and in a capsulated *S. pneumoniae*, Type III, strain A66. The uridine pyrophosphoglycosyl compound, uridine pyrophosphogalacturonic acid (UPPgalacturonic acid), has also been isolated from a capsulated *S. pneumoniae* Type I (Smith, Mills & Harper, 1957*b*). It was suggested by the above authors that such uridine derivatives function as sugar-transferring agents in the synthesis of the capsular polysaccharides of *S. pneumoniae*. Uridine pyrophosphoacetylglucosamine (UPPacetylglucosamine) has also been isolated in considerable amounts from all the above pneumococcal types, and it has been proposed (Smith *et al.* 1957*a*) that this compound is functional in synthesis of the cellular polysaccharide of pneumococci. It was therefore considered essential to investigate the structure of this polysaccharide to determine whether or not acetylglucosamine is a constituent of this material.

Information about composition of the cellular polysaccharide of pneumococci is scant. In 1930 Enders, utilizing an autolysate from a Type I pneumococcus, obtained an immune rabbit serum by repeated injections of the autolysate; when the serum was tested against the type-specific polysaccharide no precipitate was formed, but with an autolysate from smooth Type I pneumococcus the serum gave a definite precipitin reaction. An effect analogous to that described by Enders was reported by Sabin (1931), by Wadsworth &

Brown (1931) and by Ward (1932). Wadsworth & Brown (1933) separated carbohydrate fractions from pneumococci types I, II and III, and also from an atypical strain. The preparations were essentially different from the type-specific substance and were designated as cellular carbohydrate. Micro-analysis of the cellular carbohydrates yielded phosphorus values ranging from 0·43 to 6·54 % and total nitrogen values ranging from 3·03 to 8·92 %; amino-N was also shown to be present. Wadsworth, Crowe & Smith (1934) studied the absorption spectra of cellular polysaccharide preparations at different stages of purification and found that selective absorption in the ultraviolet region was characteristic of the pure substance.

Tillett & Francis (1930) isolated from a non-type-specific R strain of pneumococcus a substance called fraction C; this fraction was chemically distinct from the type-specific capsular carbohydrate and exhibited no type specificity. Tillett, Goebel & Avery (1930) studied the newly isolated fraction which was shown to contain 5·07 % nitrogen, yielded 30 % of reducing sugars on hydrolysis and contained no amino-N. The fraction exhibited a broad species coverage but no type specificity; the conclusion was that fraction C was a common constituent of all pneumococci. Heidelberger & Kendall (1931) further separated pneumococcal cellular polysaccharide into an immunologically inactive fraction and an immunologically active fraction identical with the C fraction. The serologically inactive fraction differed from fraction C in yielding on hydrolysis crystals with the optical rotation of glucosamine. The most comprehensive review of these findings is that of White (1938). The present paper describes the isolation of a polysaccharide from an extreme rough non-capsulated *Streptococcus pneumoniae* derived from a Type II organism.

METHODS

The organism used was a non-capsulated *Streptococcus pneumoniae* Type II, strain R19, kindly supplied by Dr R. D. Hotchkiss (Rockefeller Institute for Medical Research, New York, U.S.A.). Large-scale cultivation in 3·7 % (w/v) Bacto brain heart infusion medium at pH 7·4 and harvesting of the organisms in the Sharples Supercentrifuge were carried out as described by Smith *et al.* (1957*a*).

Bacto brain heart infusion (*solid*), *pepsin and trypsin* were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

Crystalline ribonuclease was obtained from Armour Laboratories, U.S.A.

Total nitrogen was determined by the micro-Kjeldahl method, using the Markham distillation unit (Ma & Zuazaga, 1942).

Glucosamine was estimated by the method of Rondle & Morgan (1955); *acetyl* groups were determined by a micro-modification of the method of Heidelberger, Kendall & Scherp (1936), which allowed determinations of the order of 0·2–1·0 mg. acetyl; *DNA-deoxyribose* estimations were carried out by the method of Ceriotti (1955) as modified by Paul (1956) and *micro-phosphorus* estimations by the method of Griswold, Humoller & McIntyre (1951). Determination of *total reducing sugar* was carried out by the method of Hagedorn &

Jensen (1923). *Paper chromatography* of sugars was carried out in the butanol/acetic acid/water solvent and the butanol/ethanol/water solvent of Partridge (1948) on Whatman no. 1 paper; the method of Consden & Stanier (1952) was employed for *ionophoresis of sugars* as borate complexes on Whatman 3MM paper strips; the aniline hydrogen phthalate reagent of Partridge (1949) was used for the development of the sugar spots. *Amino acid chromatography* was carried out on Whatman no. 1 paper using the two-dimensional butanol/acetic acid/water and phenol/ammonia system (Partridge, 1948). *Purine base and pyrimidine nucleotide chromatography* was carried out in the *iso*propanol/HCl solvent of Wyatt (1951).

RESULTS

Purification of the polysaccharide

The centrifuged organisms from 8 l. culture medium were suspended in 500 ml. distilled water, the pH value adjusted to pH 1–2 with dilute HCl and 1 g. pepsin added; digestion was allowed to proceed for 24 hr. at 37°. At the end of this period the pH value was adjusted to 9 with 2N-KOH, 2 g. trypsin added and digestion continued for a further 2 days at 37°. Tryptic activity was destroyed by neutralizing the digest and bringing it rapidly to the boil; the digest was then cooled rapidly to 30°–37°.

The pH value was readjusted to 7·8 with dilute KOH and 100 mg. crystalline ribonuclease added, followed by incubation at 37° for 3 hr. The pH value was again readjusted to 9 and a further tryptic digestion made by adding 1 g. trypsin and a 24 hr. incubation period at 37°.

At the end of this period the solution was filtered, 10 g. sodium acetate, 1 ml. acetic acid, 20 ml. chloroform and 4 ml. *n*-butanol added for every 100 ml. solution and protein removed exhaustively by the method of Sevag (1934).

The polysaccharide was precipitated from solution by the addition of 5 vol. ethanol, centrifuged and washed with 95% (v/v) ethanol in water followed by an ether washing; the material so obtained was dried *in vacuo*. The dried product was dissolved in 50 ml. water and dialysed for 2 days at 0° against several changes of distilled water. The insoluble residue obtained on dialysis was discarded and the polysaccharide was reprecipitated from solution by the addition of sodium acetate (10 g./100 ml.), acetic acid (1 ml./100 ml.) and 5 vol. ethanol. The centrifuged polysaccharide was washed with 95% (v/v) ethanol in water followed by an ether washing and was then dried *in vacuo*; yield 50 mg.

Analysis and properties of the polysaccharide

Constituent sugars obtained by acid hydrolysis. Five mg. of the isolated polysaccharide were hydrolysed with 1 ml. N-H_2SO_4 at 110° for 18 hr. and the hydrolysate neutralized with solid $BaCO_3$. The precipitated $BaSO_4$ was removed by centrifugation, washed with 1 ml. water, recentrifuged, the supernatant fluids pooled and dried *in vacuo*. The dried material was dissolved in 500 μl. distilled water, any further trace of $BaSO_4$ removed by centrifugation, and 50 μl. samples were then taken for paper chromatography and ionophoresis. As will be seen from Table 1, the constituent sugars of the isolated polysaccharide obtained by hydrolysis were galactose, mannose and glucosamine; galactose was the major component.

Table 1. *Constituent sugars of the cellular polysaccharide from* Streptococcus pneumoniae *Type II, strain R*19

R_{glucose} values by chromatography in two solvents and ionophoresis in borate buffer at pH 8·6.

	Butanol/acetic acid/water	Butanol/ethanol/water	Ionophoresis
Polysaccharide hydrolysate	0·65	0·70	0·53
	0·85	0·90	0·82
	1·26	1·24	0·91
Glucosamine	0·66	0·72	0·52
Mannose	1·27	1·27	0·81
Galactose	0·84	0·89	0·92

Trace amounts of ribose (probably from RNA contamination) were identified in both chromatographic solvents.

Glucosamine content. Fig. 1 shows the liberation of glucosamine from the polysaccharide during hydrolysis at 100° with 3N-HCl as measured by the method of Rondle & Morgan (1955); 3N-HCl gave optimal liberation of glucosamine from the polysaccharide. Glucosamine standards were run simultaneously.

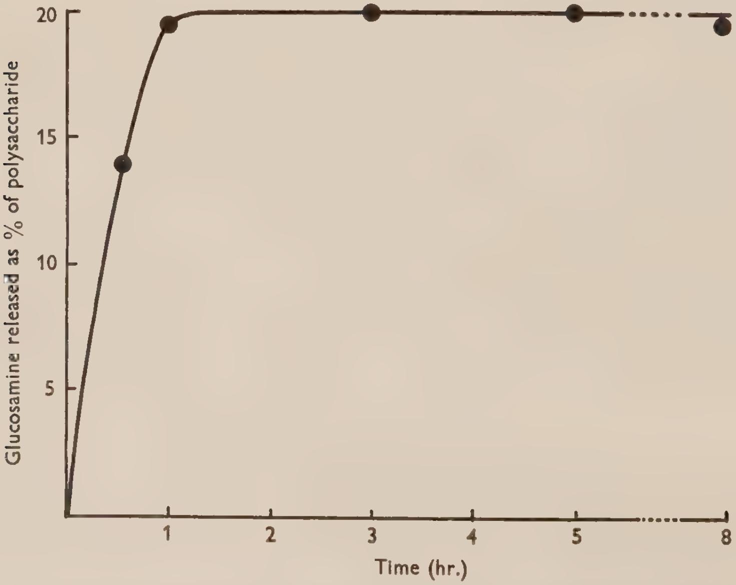

Fig. 1. Liberation of glucosamine as measured by the method of Rondle & Morgan (1955) from the polysaccharide during hydrolysis at 100° with 3N-HCl.

N-*acetyl and* O-*acetyl evaluations.* Total acetyl was determined by steam distillation of acetic acid from the polysaccharide after hydrolysis with *p*-toluenesulphonic acid. The method of Heidelberger *et al.* (1936) was modified by using a micro-distillation unit with subsequent titration of acetic acid with the Conway burette (1947). The results are recorded in Table 2. It will be observed that the acetyl content of the polysaccharide was greater than can be accounted for by *N*-acetylation of the glucosamine present; hence the following experiment was carried out to determine *O*-acetyl groups by means of their lability to alkali (Smith & Zwartouw, 1956). Six mg. polysaccharide in 1 ml. 2% (w/v) KOH were allowed to stand at room temperature for 30 min.; 10 ml. cold ethanol was added and the precipitated polysaccharide washed twice with ethanol (5 ml.). The acetyl content of the recovered polysaccharide is shown in Table 2, the results indicating that approximately 50% of the total acetyl in the polysaccharide was *N*-acetyl. The values correspond to complete acetylation of the glucosamine found in the polysaccharide.

Table 2. *Analysis of the cellular polysaccharide from* Streptococcus pneumoniae *Type II, strain R*19

	%
Total reducing sugar (calculated as glucose)	60
Glucosamine	20
Galactose + mannose (calculated)	45
Total acetyl	10·0
N-acetyl	5·3
Total nitrogen	4·8
Total phosphorus	1·3
RNA-phosphorus	0·7
DNA-phosphorus	0·05

Total reducing sugar. Hydrolysis of the polysaccharide with 3N-HCl at 100° for 3 hr. provided optimal conditions for liberation of reducing groups. Such hydrolysates were dried *in vacuo*, and total reducing sugar estimated by the method of Hagedorn & Jensen (1923). A value of 60% (calculated as glucose) was obtained; allowing for the fact that glucosamine has only 75% of the reducing power of glucose in the method used, this value would correspond to a combined galactose and mannose value of 45%.

DNA-deoxyribose estimations indicated that the polysaccharide contains 0·5% (w/v) DNA.

Ribonucleic acid content. In view of the negligible contamination of the polysaccharide with DNA, RNA contamination of the isolated material was assessed by the ultraviolet absorption spectrum, making the necessary correction for protein contamination (assuming that all the nitrogen unaccounted for by glucosamine, etc., was present as protein). When digestion with crystalline ribonuclease was omitted during the purification procedure, the polysaccharide showed 70–75% contamination with RNA; hydrolysis and chromatography confirmed the presence of adenine, guanine, uridine-5′-monophosphate and cytidine-5′-monophosphate. Inclusion of the ribonuclease digestion procedure decreased contamination with RNA to the order of 7%.

Total phosphorus. Micro-phosphorus determinations showed that the isolated polysaccharide contained 1·3% phosphorus. On the basis of 7% contamination with RNA and 0·5% contamination with DNA, the phosphorus unaccounted for by either DNA or RNA is of the order of 0·55%.

Total nitrogen. The total nitrogen present in the isolated polysaccharide was 4·8%; allowing for the nitrogen contributed by glucosamine (1·37%), RNA (0·7%) and DNA (0·05%), the residual nitrogen value is 2·68%.

Amino acid analysis. Two mg. of polysaccharide were hydrolysed with 1 ml. 6N-HCl in a sealed tube for 18 hr. at 110°, evaporated to dryness *in vacuo*, the residue dissolved in 100 μl. distilled water and chromatographed two dimensionally in butanol + acetic acid + water followed by phenol + ammonia. The following amino acids were detected: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, leucine, iso-leucine, valine, methionine, proline.

DISCUSSION

Analysis of the cellular polysaccharide from *Streptococcus pneumoniae*, Type II, strain R19, showed the constituent sugars to be galactose, mannose and acetylglucosamine. The presence of acetylglucosamine lends support to the proposal (Smith *et al.* 1957*a*) that the UPPacetylglucosamine present in *S. pneumoniae*, Types II and III, may be functional as a glycosyl donor in synthesis of the cellular polysaccharide. An extension of this investigation to the cellular polysaccharides of *S. pneumoniae*, Types I and III, has now shown that an identical sugar pattern exists for each type.

Galactose appears to be the major constituent of the sugars obtained on acid hydrolysis of the polysaccharide; this is of interest in view of the fact that the UPPglucose isolated from *Streptococcus pneumoniae*, Type II, strain R19, has been found to be a mixture of UPPglucose and UPPgalactose (unpublished observation).

During the isolation of the cellular polysaccharide it was found that RNA was a major contaminant unless a ribonuclease digestion were included in the purification procedure. This may account for the observation of Wadsworth *et al.* (1934) that selective absorption in the ultraviolet region was characteristic of the 'pure' substance. The different phosphorus values of previous preparations (0·43–6·54%) may also be accounted for by the contaminating RNA. The residual 7% RNA associated with the present preparation can probably be attributed to the residual core of RNA which is not attacked by ribonuclease.

A considerable amount of nitrogen (2·68%) is unaccountable for in terms of glucosamine-N, RNA-N or DNA-N. From the amino acid pattern obtained it seems probable that this nitrogen is associated with protein. Attempts to remove this protein by repeated Sevag treatments and fractional alcohol precipitation of the purified polysaccharide have so far failed, nor have such procedures led to any change in the amino acid pattern.

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REFERENCES

Ceriotti, G. (1955). Determination of nucleic acids in animal tissues. *J. biol. Chem.* **214**, 59.

Consden, R. & Stanier, W. M. (1952). Ionophoresis of sugars on paper and some applications to the analysis of protein-polysaccharide complexes. *Nature, Lond.* **169**, 783.

Conway, E. J. (1947). *Micro-Diffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby-Lockwood.

Enders, J. F. (1930). A type specific substance distinct from the specific carbohydrate in pneumococcus Type I. *J. exp. Med.* **52**, 235.

Griswold, B. L., Humoller, F. L. & McIntyre, A. R. (1951). Inorganic phosphates and phosphate esters in tissue extracts. *Analyt. Chem.* **23**, 192.

Hagedorn, H. C. & Jensen, B. N. (1923). Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Z.* **135**, 46.

Heidelberger, M. & Kendall, F. E. (1931). Specific and non-specific polysaccharides of Type IV pneumococcus. *J. exp. Med.* **53**, 625.

Heidelberger, M., Kendall, F. E. & Scherp, H. W. (1936). The specific polysaccharides of Types I, II and III pneumococcus. A review of methods and data. *J. exp. Med.* **64**, 559.

Ma, T. S. & Zuazaga, G. (1942). Micro-Kjeldahl determination of nitrogen. A new indicator and an improved rapid method. *Industr. Engng Chem.* (*Anal.*), **14**, 280.

Partridge, S. M. (1948). Filter paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

Partridge, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **164**, 443.

Paul, J. (1956). The chemical determination of deoxyribonucleic acid in tissue cultures. *J. Biophysic. Biochem. Cytol.* **2**, 797.

Rondle, C. J. M. & Morgan, W. T. J. (1955). The determination of glucosamine and galactosamine. *Biochem. J.* **61**, 586.

Sabin, A. B. (1931). On the presence in antipneumococcus serum of type-specific protective antibody not neutralized by homologous specific soluble substance. *J. exp. Med.* **53**, 93.

Sevag, M. G. (1934). Eine neue physikalische Enteiweisungsmethode zur Darstellung biologisch wirksamer Substanzen. *Biochem. Z.* **273**, 419.

Smith, E. E. B., Mills, G. T. & Harper, E. M. (1957*a*). A comparison of the uridine pyrophosphoglycosyl metabolism of capsulated and non-capsulated pneumococci. *J. gen. Microbiol.* **16**, 426.

Smith, E. E. B., Mills, G. T. & Harper, E. M. (1957*b*). The isolation of uridine pyrophosphogalacturonic acid from a Type I pneumococcus. *Biochim. biophys. Acta*, **23**, 662.

Smith, H. & Zwartouw, H. T. (1956). The polysaccharide from *Bacillus anthracis* grown *in vivo*. *Biochem. J.* **63**, 447.

Tillett, W. S. & Francis, T. Jun. (1930). Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J. exp. Med.* **52**, 561.

Tillett, W. S., Goebel, W. F. & Avery, O. T. (1930). Chemical and immunological properties of a species-specific carbohydrate of pneumococci. *J. exp. Med.* **52**, 895.

Wadsworth, A. & Brown, R. (1931). Chemical and immunological studies of the pneumococcus. I. A specific antigenic carbohydrate of Type I pneumococcus. *J. Immunol.* **21**, 245.

Wadsworth, A. & Brown, R. (1933). Chemical and immunological studies of the pneumococcus. III. Cellular carbohydrate fractions. *J. Immunol.* **24**, 349.

Wadsworth, A., Crowe, M. O'L. & Smith, L. A. (1934). Absorption spectra of the carbohydrates of the pneumococcus. A preliminary note. *J. Immunol.* **26**, 481.

Ward, H. K. (1932). An examination of the mechanism of pneumococcus immunity by means of bactericidal measurements. II. The reaction between the anti-carbohydrate antibody and type-specific products of the organism. *J. exp. Med.* **55**, 519.

White, B. (1938). *Biology of the Pneumococcus.* New York: Commonwealth Fund.

Wyatt, G. R. (1951). Recognition and estimation of 5-methyl cytosine in nucleic acids. *Biochem. J.* **48**, 581.

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Steel, R. & Walker, T. K. (1957). *J. gen. Microbiol.* **17**, 445–452

A Comparative Study of Cellulose-Producing Cultures and Celluloseless Mutants of certain *Acetobacter* Spp.

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SUMMARY: Comparative studies have been conducted with two strains of *Acetobacter acetigenum* (NCIB 8132, NCIB 5346), one strain of *A. xylinum* var. *africanum* (NCIB 7029) and a total of 26 celluloseless mutants obtained from these three parent cultures. In addition to the loss of ability to produce a cellulose pellicle, other consistent differences were also observed between the parent and the mutant cultures. Whereas the parent cultures oxidized ethanol to acetic acid this reaction was not observed with mutant organisms. The mutants showed (1) optimum growth in alkaline media, (2) growth in glucose yeast-extract medium in the presence of 10–12 % (v/v) ethanol, (3) proteolytic activity, (4) growth at 40°, and (5) growth in yeast-extract medium; these abilities were not shown by the wild-type cultures. Variations in colony form, nutritional requirements, the ability to produce acid from certain sugars and to oxidize glycerol appeared to occur at random among the mutant cultures.

In their paper about 'celluloseless mutants' of *Acetobacter xylinum*, Schramm & Hestrin (1954) did not mention any other differences between these mutants and the wild-type cellulose-producing culture. On the other hand, the cultures of 'celluloseless' mutants examined by Steel & Walker (1957) differed from the wild-type cultures in colony form and in growth rate on glucose yeast-extract agar medium. The present work was undertaken to determine other differences or similarities between the cellulose-producing organisms and the celluloseless mutants obtained from them.

METHODS

Cultures studied and cultural conditions. The cellulose-producing cultures which were examined were two strains of *Acetobacter acetigenum* (NCIB 8132 and NCIB 5346) and one strain of *A. xylinum* var. *africanum* (NCIB 7029) obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex. Cultures of celluloseless mutants were obtained from these three parent cultures by methods described previously (Steel & Walker, 1957). In all, a total of 26 mutant cultures was obtained by eight different methods (Table 1). Six of the mutant isolates were obtained from organism NCIB 8132 when it lost the ability to produce a cellulose pellicle during the course of the present work. Three of the mutant cultures died during the present study; apparently the cultures should be transferred at weekly intervals to ensure their viability. One other culture reverted to cellulose-formation after it had failed to produce cellulose pellicles in 4 subcultures (2 weeks).

Unless otherwise stated, all studies were made according to procedures outlined by Conn (1949). Glucose yeast-extract (GYE) medium (pH 7·0) contained glucose 3% (w/v) and Difco yeast-extract 0·5% (w/v) in distilled water; for solid media, agar (2%, w/v) was added. Malt wort medium was prepared by the method of Tosic & Walker (1946). The parent cultures were kept in malt wort medium since certain cultures lost their ability to produce a cellulose pellicle when grown continuously in GYE medium (Steel & Walker, 1957). The mutant organisms were stored on GYE medium because certain of these cultures failed to grow in malt wort medium (see later). With the exceptions mentioned later, media were sterilized in 8 ml. amounts in 18 × 155 mm. test tubes for 10 min. at 15 lb./sq.in. pressure; inoculations were made by wire loop from 48 hr. cultures in GYE medium and incubation was at 30° unless otherwise stated.

Table 1. *Sources of mutant cultures of* Acetobacter *spp. studied*

Method by which obtained*	Parent culture	No. of mutant cultures selected for study
Glucose yeast-extract (GYE) medium, SH†	*A. acetigenum* NCIB 8132	5
Ethanol yeast-extract (EYE) medium, SH‡	*A. acetigenum* NCIB 8132	3
Selective medium + ethanol, ST§	*A. acetigenum* NCIB 8132	1
Ethyleneglycol + yeast-extract medium, ST	*A. acetigenum* NCIB 8132	1
EYE medium, ST‡	*A. acetigenum* NCIB 8132	1
GYE medium, mass plating	*A. acetigenum* NCIB 8132	2
Selective medium, replica plating	*A. acetigenum* NCIB 8132	1
GYE medium, ST	*A. acetigenum* NCIB 8132	6
GYE medium, ST	*A. acetigenum* NCIB 5346	3
GYE medium, ST	*A. xylinum* var. *africanum* NCIB 7029	3
	Total	26

* Indicates the conditions under which the parent culture was grown (cf. Steel & Walker, 1957).

† SH = shaken culture.

‡ EYE medium containing ethanol (20%, v/v).

§ ST = static culture.

Frateur's tests. The 'Oxydogramme' tests recommended by Frateur (1950) for the classification of *Acetobacter* species were followed. Briefly, washed organisms (washed pellicles in the cases of the parent cultures) obtained from 48 hr. GYE cultures were incubated on the surface of sterile agar media containing the desired substrate (2%, w/v or v/v, for solids and liquids, respectively). Acid production from ethanol and from glucose was detected by the dissolution of calcium carbonate (2%, w/v) present in the medium. Subsequent oxidation of the acetic acid produced from ethanol was shown by the reprecipitation of calcium carbonate on further incubation. Oxidation of calcium lactate to acetate is shown by the formation of a clear zone surrounding the added bacteria. The oxidation of glycerol to dihydroxyacetone was observed by the precipitation of copper oxide when the plates were flooded with Fehling's solution. Catalase was detected by the evolution of oxygen when Petri dishes containing the desired culture (GYE medium) were flooded with 2% (v/v) hydrogen peroxide (100 vol.) in distilled water.

Nutritional studies. The quantities (%, w/v, in distilled water) of various ingredients used in defined media were as follows: glucose, 3·0; $(NH_4)_2SO_4$, 0·3; KH_2PO_4, 0·3; $MgSO_4.7H_2O$, 0·2; Casamino acids (Difco), 1·0; biotin, 0·00003; riboflavin, 0·0002; calcium pantothenate, 0·0002. These vitamins were used previously by Creedy, Jowett & Walker (1954) for the cultivation of *Acetobacter acetigenum*. The media (pH 5·9) were sterilized in 6 ml. amounts in nephelometer tubes. Mutant organisms were transferred from 48 hr. GYE agar-slope cultures to 10 ml. sterile distilled water and one drop of this suspension (*c.* 0·05 ml.) was used as inoculum for each tube of test medium. In the case of parent cultures, a 48 hr. pellicle, washed with four changes of sterile distilled water, was shaken in the test medium as an inoculum. Each culture was tested in duplicate. Growth was assessed by measurement of the turbidity with a nephelometer or, in the case of cellulose-producing cultures, by visual assessment. The cultures were incubated for 7 days.

Production of acid and gas from sugars. The basal medium (pH 7·0) consisted of yeast-extract (Difco, 0·5 %, w/v) + bromcresol purple (0·003 %, w/v) in distilled water; the test sugars were added to 1 % (w/v). Each tube of medium contained an inverted Durham tube to test for gas production. The cultures were examined daily for 8 days.

Optimum pH *range for growth.* Growth was assessed after 3 days' incubation in GYE medium at initial pH values ranging from 4·0 to 9·0 in increments of 0·5 pH units. Also, growth curves of certain mutant cultures (initial pH 7·0) were obtained by measurement of the turbidity with a nephelometer.

Tolerance to ethanol. These tests were made in GYE medium containing from 2 to 14 % (v/v) ethanol (95 %) in increments of 2 %; sterile ethanol was added aseptically to sterile GYE medium.

Growth at 40°. After inoculation into GYE medium, the cultures were incubated at 40° for 5 days.

Growth in milk. A sample of skim milk (pH 6·5) was sterilized with free steam for 30 min. on 3 consecutive days before inoculation with the test organisms; incubation was for 14 days.

Liquefaction of gelatin. The test medium was gelatin (12 %, w/v) + yeast extract (Difco, 0·5 %, w/v) in distilled water. Organisms from GYE agar slant cultures (4 days) were used as inoculum; incubation was at room temperature (*c.* 18°) for 4 weeks.

Production of ammonia and hydrogen sulphide. The growth medium (pH 7·0) was Evans's peptone (2 %, w/v) + yeast extract (Difco, 0·5 %, w/v) in distilled water. After 3 days of incubation the cultures were boiled in the presence of litmus paper to detect ammonia; hydrogen sulphide was detected by the method of Morse & Weaver (see Conn, 1949).

Production of indole and reduction of nitrate. The test media (aqueous) contained peptone (2 %, w/v), yeast extract (0·5 %, w/v) and either casein (fat free, Hopkin and Williams Ltd.) (0·5 %, w/v) or potassium nitrate (0·1 %, w/v) respectively, depending whether the test was to determine indole production or nitrate reduction. The cultures were tested at 5 days.

RESULTS

During the present work no marked differences were found between the different species of wild-type organisms examined; in fact, there was as much variability in properties between the two different strains examined as there was between the two different species (see Table 4). Also, the mutant organisms obtained from the different strains and species of wild-type cultures were indistinguishable. For these reasons the results are presented collectively.

Description of colonies. On GYE agar medium colonies of organisms NCIB 8132, NCIB 5346 and NCIB 7029 were punctiform, 1 mm. or less in diameter, pale brown in colour, convex, with a smooth semi-matt to shiny surface, regular edge, and tough consistency; the whole colony lifted off the agar when touched with a needle; the colonies appeared on the plates after 3–5 days of incubation (Pl. 1, fig. 1). At 24 hr. colonies of all mutant cultures were 2–4 mm. in diameter, white, circular, flat, with a diffuse edge, butyrous consistency, smooth and semi-matt surface showing lighter and darker areas (Pl. 1, fig. 2). At 48 hr. the colony form of 21 of the 26 cultures had changed to that shown in Pl. 1, fig. 3, which afforded the following description: colonies 3–5 mm. in diameter, circular, white to cream-coloured, raised, regular edge, smooth and glistening surface, butyrous consistency. Three of these mutant cultures sometimes showed a different colony form after 4–6 days of incubation (Pl. 1, fig. 4). The five remaining cultures gave colonies at 48 hr. which were 2–5 mm. in diameter, white, circular, convex, with a semi-matt surface and butyrous consistency; secondary colonies were produced by two of these cultures after 5–7 days' incubation (Pl. 1, figs. 5, 6). Yellow pigmentation was produced by one mutant culture, and three others showed an area in the centre of the colony suggestive of autolysis. Both of these latter characteristics only appeared in old cultures (10–14 days) and were variable.

Morphology, size, staining reaction and motility. Vegetative organisms of parent and mutant cultures were Gram-negative motile rods when cultivated for 24 hr. at 30° in GYE medium; involution forms were also present in most cultures. All isolates examined were aerobic. The majority of mutant organisms were within the range 0·4–0·8 μ. wide × 0·6–2·0 μ. long when stained with methylene blue. Spore formation was not observed with any of the isolates studied. Capsule formation was observed with the parent cultures when grown for 24 hr. in GYE liquid medium, whereas only some of the mutant isolates produced a capsule when grown under these conditions. When grown for 24 hr. on GYE agar medium, all mutants showed capsules.

Growth in GYE medium. Growth in GYE medium was similar to that described previously (Steel & Walker, 1957). Briefly, parent cultures produced a surface pellicle of cellulose within 48 hr. incubation and the medium under the pellicle remained virtually clear; in contrast, the mutants grew diffusely throughout the medium within 24 hr. of incubation and did not produce a cellulose pellicle. Two of the mutant cultures generally showed abundant 'rope' formation. Growth curves of 9 out of 11 mutant isolates examined on GYE medium were of the diauxic type (Monod, 1947).

Growth in malt wort medium. Of 7 mutant isolates tested, 3 did not grow in malt wort medium; the 3 parent cultures grew well.

Growth on GYE agar slopes. At 3–5 days of incubation, growth of the parent cultures was of the beaded type. At 24 hr. of incubation growth of mutant cultures was moderate, white, filiform, glistening and butyrous. Differences in growth type between the various mutant isolates were very slight. After prolonged incubation (10 days) eleven of the mutant isolates produced a yellow pigment; this, however, was a variable property.

Frateur's tests. A summary of the results obtained by using the tests recommended by Frateur (1950) is given in Table 2. All parent and mutant isolates produced catalase but did not oxidize calcium lactate. The parent cultures oxidized ethanol, acetate, glycerol (ketogenic activity) and glucose. In contrast, the mutant isolates showed no evidence of acid production from ethanol or glucose, while ketogenic activity was shown only by certain of these cultures. To obtain a positive reaction in the latter test it was necessary to incubate mutant organisms with substrate for 24–48 hr. before adding Fehling's solution; the recommended incubation period (Frateur, 1950) of 1–2 hr. gave positive results with parent cultures only. The positive reactions given by the majority of the mutant isolates were very weak in comparison with those given by the parent cultures. In other experiments with a Warburg respirometer (Umbreit, Burris & Stauffer, 1945), suspensions of two mutant isolates examined showed no oxygen uptake with ethanol as substrate whereas oxygen consumption with glucose was quite rapid.

Table 2. *Proportion of cultures tested which gave a positive reaction with Frateur's tests (Oxydogramme method)*

	Cultures	
	Parent	Mutant
Catalase present	3/3	26/26
Oxidation of calcium lactate	0/3	0/26
Oxidation of ethanol and acetate	3/3	0/26
Oxidation of glycerol to dihydroxyacetone	3/3	14/26
Oxidation of glucose to gluconic acid	3/3	0/26

Nutritional studies. A summary of these findings is given in Table 3. The parent cultures grew in glucose + salts medium and at a faster rate when vitamins were added; however, they did not grow in the Casamino acids + salts medium. The nutritional requirements were variable among the mutant isolates; 16 of 21 isolates showed either poor or moderate growth in glucose + salts medium and, in general, the same isolates showed a similar amount of growth when vitamins were added. Casamino acids + salts medium supported the growth of 17 of 21 mutant cultures, but the addition of glucose to this medium did not appreciably affect the amount of growth obtained. Only one mutant isolate did not grow in the medium containing all components; this culture grew moderately well in GYE medium.

Table 3. *Proportion of cultures which grew in certain defined media*

Medium	Cultures Parent	 Mutant
Glucose, salts	3/3	16/21
Casamino acids, salts	0/3	17/21
Glucose, salts, vitamins	3/3	17/21
Glucose, salts, Casamino acids	3/3	19/21
Glucose, salts, vitamins, Casamino acids	3/3	20/21

Production of acid and gas from sugars. It may be noted from the results (Table 4) that acid was produced from more sugars by the mutant than by the parent cultures; however, acid was not produced by all of the mutant cultures from any one sugar. Lactose was the only sugar from which acid was not produced by any mutant. It is noteworthy that certain mutant isolates which had given a negative result by Frateur's method (Table 2) produced acid from glucose by this method of test. Gas production was not observed.

Table 4. *Proportion of cultures tested which produced acid from certain sugars*

	Cultures Parent	 Mutant
Xylose	2/3*	8/18
Arabinose	1/3†	9/18
Glucose	3/3	14/18
Fructose	0/3	14/18
Galactose	0/3	8/18
Lactose	0/3	0/18
Sucrose	0/3	14/18
Maltose	0/3	2/18

* Acid produced by cultures NCIB 8132 and NCIB 7029.
† Acid produced by culture NCIB 8132.

Table 5. *Reaction of parent and of mutant cultures to certain tests*

	Cultures Parent	 Mutant
Optimum pH range for growth	5·0–6·0	7·5–8·5
Ethanol tolerance	4 %, (v/v)	10–12 %, (v/v)
Gelatin liquefaction	0/3*	26/26
Growth at 40°	0/3	9/9
Growth in yeast extract (0·5 %, w/v) medium	0/3	26/26
Milk peptonization	0/3	22/23
Ammonia production	0/3	16/26
Hydrogen sulphide production	0/3	0/26
Indole production	0/3	0/26
Nitrate reduction†	0/3	0/26

* Proportion of isolates tested which gave a positive reaction.
† A positive test for nitrite after the addition of zinc dust indicated that nitrate was present.

Additional experiments. The results of other experiments (Table 5) revealed that, unlike the parent cultures, the mutants (1) showed optimum growth at alkaline pH values, (2) tolerated 10–12% (v/v) ethanol, (3) liquefied gelatin, (4) grew at 40°, and (5) grew in yeast-extract medium; in addition, most of the mutant isolates peptonized milk or produced ammonia. None of these abilities were shown by the parent cultures. Furthermore, none of the isolates tested (parent or mutant) produced hydrogen sulphide or indole or reduced nitrate. The adequacy of these test procedures was confirmed by using an organism which gave positive reactions under the conditions of test.

DISCUSSION

That the mutant isolates studied differ from the parent cultures in several respects is clear (Tables 2–5). Some of the differences observed between the parent and the mutant cultures were common to all parent and mutant isolates examined. For example, unlike the parent cultures, the mutants did not oxidize ethanol to acetic acid, showed optimum growth at alkaline pH values, were able to tolerate high concentrations of ethanol, liquefied gelatin, grew at 40° and grew in yeast extract medium. In contrast to these associated changes, the differences observed in colony types (secondary colonies), nutritional requirements, oxidation of certain sugars and glycerol, probably represented independent changes since they occurred at random among the mutant cultures. It is also possible that some of the observed differences in nutritional requirements arose from an imbalance of amino acids supplied (cf. Gladstone, 1939).

In contrast with the results of Frateur (1950) is the fact that the oxidation of calcium lactate by the parent cultures was not demonstrated in the present work.

The failure to oxidize ethanol to acetic acid would appear to exclude the mutants as belonging to the genus *Acetobacter* (*Bergey's Manual*, 1948). Moreover, proteolytic activity is not found among members of this genus (Frateur, 1950). Apart from minor differences, the mutant organisms bear a resemblance to the description given for *Pseudomonas geniculata* in *Bergey's Manual of Determinative Bacteriology*. Our attempts to isolate, from samples of raw vinegar, bacteria which exhibit properties similar to those shown by the mutant organisms have been unsuccessful (results not given in the text).

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REFERENCES

Bergey's Manual of Determinative Bacteriology (1948), 6th ed. Edited by Breed, R. S., Murray, E. G. D. & Hitchens, A. P. Baltimore: The Williams and Wilkins Co.

Conn, H. J. (1949). *Manual of Methods for Pure Culture Study of Bacteria.* Geneva (New York, U.S.A.): Biotech. Publications.

Creedy, A. E., Jowett, P. & Walker, T. K. (1954). Formation of D-cellobiose from starch and from other substrates by an *Acetobacter* species. *Chem. & Ind.* (*Rev.*), **73**, 1279.

Frateur, J. (1950). Essai sur la systematique des acetobacters. *La Cellule*, **53**, 287.

Gladstone, G. P. (1939). Inter-relationships between amino acids in the nutrition of *B. anthracis*. *Brit. J. exp. Path.* **20**, 189.

Monod, J. (1947). The phenomenon of enzymic adaptation and its bearing on problems of genetic and cellular differentiation. *Growth*, **11**, 223.

Schramm, M. & Hestrin, S. (1954). Factors affecting production of cellulose at the air-liquid interface of a culture of *Acetobacter xylinum*. *J. gen. Microbiol.* **11**, 123.

Steel, R. & Walker, T. K. (1957). Celluloseless mutants of certain *Acetobacter* species. *J. gen. Microbiol.* **17**, 12.

Tosic, J. & Walker, T. K. (1946). Procedure for the characterisation of the acetic acid bacteria. *Chem. & Ind.* (*Rev.*), **65**, 104.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Tissue Metabolism.* Minneapolis (Minnesota, U.S.A.): Burgess Publishing Co.

EXPLANATION OF PLATE

(Magnification, × 10.)

Fig. 1. Colonies of cellulose-producing organisms.

Figs. 2–6. Colonies of celluloseless-mutant organisms (see text for details).

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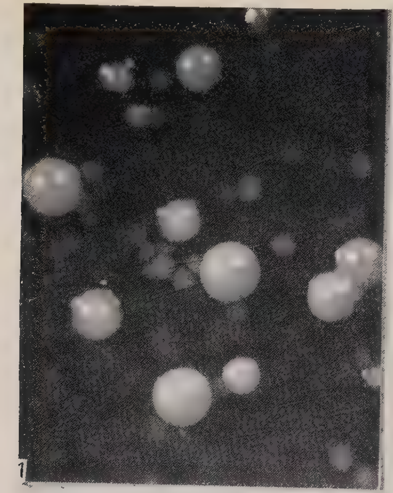
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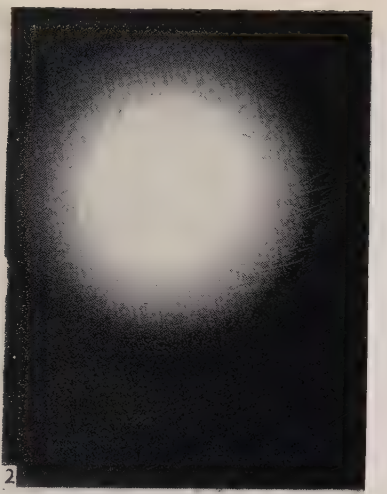
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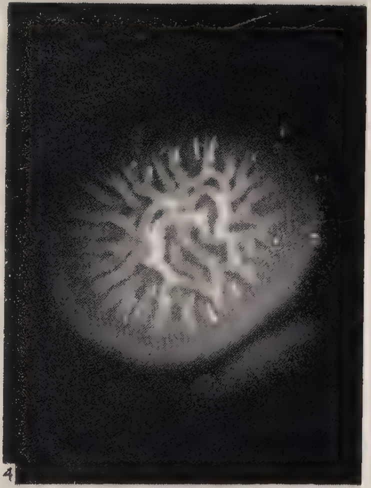
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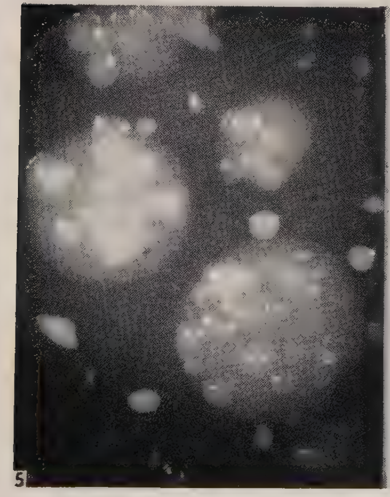
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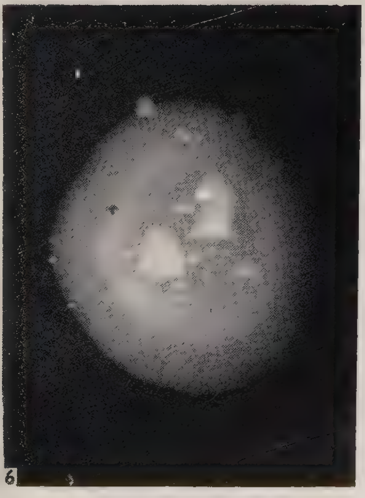
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R. STEEL & T. K. WALKER—COMPARATIVE STUDIES OF *ACETOBACTER* SPP. PLATE 1

(*Facing p.* 452)

WHITFIELD, J. F. & APPLEYARD, R. K. (1957). *J. gen. Microbiol.* 17, 453–466

Formation of the Vegetative Pool by Induced Defective and Healthy Lysogenic Strains of *Escherichia coli*

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SUMMARY: Comparison of two defective λ prophages carried by strains of *Escherichia coli*, with respect to their physiological properties following induction, has revealed that one defective mutant ($\lambda\,i2$) can multiply normally as vegetative phage but cannot mature into normal infectious phage, whereas the other defective phage ($\lambda\,i1$) can undergo only limited multiplication since it is unstable in the vegetative state. Consideration of the properties of $\lambda\,i1$ has led to an hypothesis that radiation induction causes the prophage to produce a vegetative replica which becomes autonomous rather than conversion of the prophage into vegetative phage.

Lysogenic strains of *Escherichia coli* containing defective forms of the bacteriophage λ have been investigated by Appleyard (1954, 1956) and by Jacob & Wollman (1956). They are distinguished by an almost total failure to liberate healthy phage following induction, and Appleyard (1956) showed that the defect may be transmitted by extracellular defective phage. The phage genes responsible for the defects resemble the recessive lethal genes of higher organisms; induction by ultraviolet irradiation of a doubly lysogenic strain, in which a normal and a defective prophage are present in the same cell, is followed by liberation at lysis of healthy phage particles, some of which have acquired genes from the defective prophage by recombination during the latent period.

As with recessive lethal genes, phage defects of independent origin would only rarely be expected to be genetically or physiologically identical. Appleyard & Whitfield (1956) demonstrated recombination between two such defects to give rise to normal progeny. Therefore, they are not allelic but are genetically distinct factors. In the present study the physiological effects of these defects have been compared.

METHODS

Bacteria and bacteriophages

We used as experimental material strains of *Escherichia coli* and bacteriophages described in earlier papers from this laboratory (Appleyard, 1956; Appleyard, McGregor & Baird, 1956). The two defects of independent origin chosen for investigation were those originally isolated in strains C33 ($\lambda\,i2$) and C60 ($\lambda\,i1$) (Appleyard, 1954). Both defects can be transmitted through extracellular phage to the same sensitive bacterium (Appleyard, 1956) with the advantage that observed physiological differences cannot then be due to strain differences between the bacterial hosts in which the defects were originally found. The cultures C112 ($\lambda\,i1$) and C112 ($\lambda\,i2$) as well as the healthy lysogen

C112 (λ *s*) used in our experiments were prepared by artificial lysogenization of the same sensitive strain C112, a descendant of strain 12–112 of Wollman (1953). Bacteriophage λ-*s* was chosen as the healthy control phage, since both it and $\lambda\,i1$ and $\lambda\,i2$ were derived from the same ancestral strain of *E. coli* K12 (λ).

Production of healthy phage by ultraviolet-stimulated reverse mutation

If the defects arise as mutations of phage genes, many of them should be reversible through 'back mutation'. To study the frequency of u.v. stimulated reversion at various steps of phage development, a culture of defective lysogenic organisms was first induced at time zero by an exposure to a small dose of u.v. radiation. The induced organisms were allowed to develop and at various times during development several portions of the induced culture were exposed to different doses of u.v. radiation and after each dose the bacteria yielding healthy phage were counted. At any given time, the slope of the mutation/dose curve near the origin was taken as a measure of mutability. The detailed procedure was as follows.

Defective lysogens were prepared for induction by one of two methods:

(1) Organisms were grown on the surface of nutrient agar plates and, while still in the logarithmic phase of growth, were washed off in ice-cold buffer and diluted to a turbidity value (nephelometrically determined) which corresponded to a concentration of 3×10^8 viable organisms/ml.

(2) Organisms were grown in liquid Tryptone (Difco) broth with aeration to a concentration of 3×10^8 viable organisms/ml. (just out of the logarithmic phase of growth). They were then separated from the broth by centrifugation at 4° for 3 min. at 8000 ***g*** and resuspended in an equal volume of ice-cold buffer. In either case the organisms were at once exposed to u.v. radiation (2537A) for 15 sec. (225 ergs/mm.^{2}) to induce them. Tryptone was added to a concentration of 1% (w/v) (1 ml. of a 10% (w/v) solution of Tryptone to 9 ml. suspension of organisms) and the culture was aerated at 37°.

At each time to be investigated, the organisms in 5 ml. of the culture were spun down and resuspended in an equal volume of ice-cold buffer as before. Separate 1 ml. portions of this buffer suspension were exposed to a further dose of u.v. radiation usually for 20, 40 and 60 sec., and were at once assayed, with an unirradiated control, as plaque-formers before lysis by the agar layer technique.

Recombinational analysis of pool size

It is possible to superinfect an induced lysogenic organism with a phage which has been suitably marked genetically, and to relate the number of superinfecting normal phage/induced lysogenic organism and the proportion of recombinant phage released at lysis to the genetic or vegetative pool size (Visconti & Delbrück, 1953) in the induced lysogenic organism at the time of superinfection. This relation will be described in full in the experimental section of the present paper. To determine the proportion of recombinants produced by superinfection following induction, cultures of C112 (λ *s*), C112

(λ *i*1) and C112 (λ *i*2) were grown to 3×10^8 viable organisms/ml. in Tryptone broth. Ten ml. culture were spun down at 7000 ***g***. The organisms were resuspended in 9 ml. buffer and u.v.-irradiated (225 ergs/mm.^{2}). One ml. of a 10% (w/v) solution of Tryptone in distilled water was added to the induced culture, which was then aerated at 37°. After various time intervals, 0·9 ml. samples of the culture were removed and transferred to a tube containing 0·1 ml. at various titres of phage λ *v*1 *h* (clear-plaque former) a host-range mutant of the weak virulent λ phage (Appleyard, 1954; Appleyard *et al.* 1956). Beginning at any time between 0 and 45 min. after induction of the lysogenic host organisms 99·9% of the phage λ *v*1*h* was adsorbed within 15 min. at 37°. This fraction remained the same in mixtures containing up to 60 phage particles/organism.

Each infected culture was aerated until 30 min. after the appearance of visible lysis. The lysates were then shaken with 0·1 ml. chloroform/ml. lysate, diluted 1/100 in broth and assayed by the double layer method on plates containing medium to a depth of not less than 1 cm. (Jacob & Wollman, 1954) to determine the numbers of turbid (recombinant) and clear plaques (normal parental phenotype).

In order to study changes in the normal healthy lysogenic strain of *Escherichia coli* C112 (λ *s*) following induction, a slightly different procedure was followed. The lysates were diluted and mixed with sufficient organisms from a freshly grown culture of the sensitive strain CR600 (Appleyard *et al.* 1956) to obtain a phage to bacterium ratio of at most 1:100. The infected culture was plated before lysis on the host-range indicator strain *E. coli* CR63. This method of pre-adsorption eliminates any distortion of the data due to phenotypic mixing (Appleyard *et al.* 1956) as well as improving the clarity and size of the plaques. A phage to bacterium ratio of 1:100 was used to avoid double infection, which would also distort the results.

RESULTS

Physiological comparisons between lysogens carrying defects i1 *and* i2

The defects show superficial similarities. Both are transferable by infection and can therefore be studied in bacteria having otherwise identical genotypes. Mass lysis of bacteria carrying either defective prophage can be induced by small doses of u.v. radiation. It occurs 85 min. after exposure to u.v. light with liberation of an average of one healthy phage/10^5 to 10^6 induced organisms. In both defective lysogens the production of infective phage, i.e. 'reverse mutants', can be stimulated by exposure to further doses of u.v. radiation during development, an average of 6 to 10 phage particles being released from each exposed organism.

Differences in frequency of u.v.-induced reversions of i *to* i$^+$

A marked difference between the two defects was observed with respect to frequency of reverse mutant phage detected following the double irradiation technique described above (Fig. 1). Following induction of organisms of

Escherichia coli C112 ($\lambda\ i2$) in the logarithmic phase of growth, the frequency of reversions was over 10 times as great when the second irradiation was applied at 30 min. as when the second irradiation was applied at 0 min. (Fig. 1A). After 30 min., the frequency dropped sharply. In log phase organisms of C112 ($\lambda\ i1$), no such increase was observed (Fig. 1A). Although the variation in time of the u.v. stimulation was somewhat different for older cultures (phase of retarded growth) the marked contrast in behaviour between $i1$ and $i2$ remained (Fig. 1B). These observations suggested to us that the number of $\lambda\ i2$ vegetative phage units increases with time following induction, whereas the number of $\lambda\ i1$ vegetative phage units does not. To investigate this possibility it was necessary to devise ways of measuring the size of the vegetative or genetic pool in the induced defective and normal lysogens.

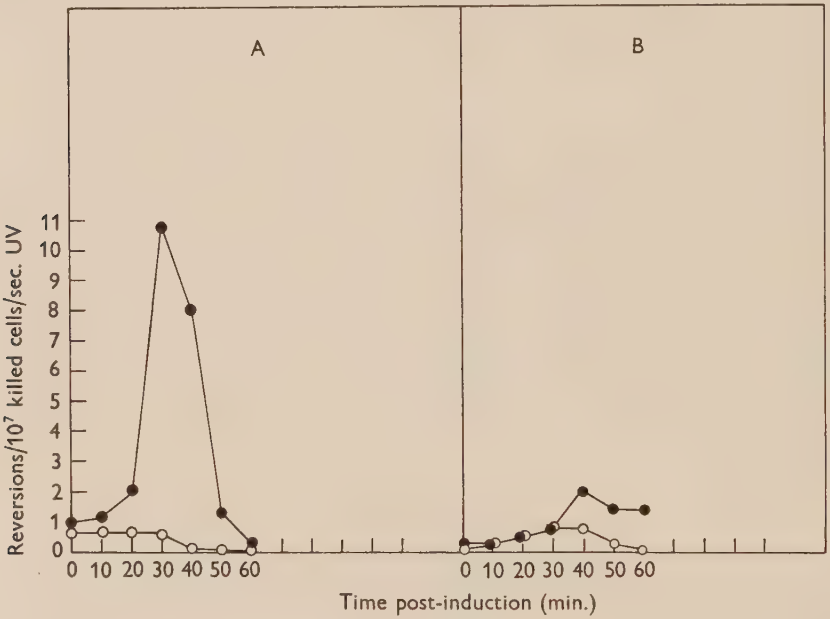

Fig. 1. The variation with time of the u.v. stimulated production of healthy phage following induction of *Escherichia coli* C112 ($\lambda\ i1$) (○—○) and *E. coli* C112 ($\lambda\ i2$) (●—●), (A) from cultures in the logarithmic phase of growth, (B) from cultures in the retarded phase of growth.

Recombinational estimate of pool size

Principles of experiment. By the genetic or vegetative pool we mean the total number of actively-recombining replicas of the phage genotype inside the organism (Visconti & Delbrück, 1953). The simplest method of estimating the pool size is to measure the number of recombining units by some kind of superinfection with a genetically marked phage, and experiments on these lines have been carried out by a number of workers (Levinthal & Visconti, 1953; Visconti & Garen, 1953; Jacob & Wollman, 1956). It is clear from the work of Jacob (1954), as well as from our own experiments, that the recombinational method includes the prophages in its measure of the pool size. It therefore strictly measures the genetic pool size rather than the vegetative. It was, however, necessary to avoid possible complications connected with selection for or against recombinants, with variations of the recombination process as a function either of time of irradiation (Jacob & Wollman, 1955) or of the physiological state of the organisms in which defective development was proceeding. Moreover, we were unable to measure the total yield because it included defective phage. We therefore used a variation of the simple method based on the following argument.

Let m be the number of defective, recombinable phage replicas/induced organism and let n be the number of superinfecting healthy phage particles/organism. If the ratio of m to n be constant throughout the remainder of the latent period, then the number of recombinants, N, issuing from such an infection should be directly proportional to the fraction of matings in which recombination is possible. If matings are at random, this fraction is $2mn/(m+n)^2$.

Then,

$$N=\frac{2Kmn}{(m+n)^2}. \tag{1}$$

Since the numbers of reciprocal recombinants are statistically equal in infections with λ phage (Jacob & Wollman, 1954; Wollman & Jacob, 1954), the fraction of viable recombinants in the yield will be some constant fraction of N

$$N'=\frac{K'mn}{(m+n)^2}, \tag{2}$$

whereas the total fraction of viable phage particles will be $n/(m+n)$.

Therefore, the proportion, p, of recombinants among the total yield of active phage particles will be

$$p=\frac{K'm}{(m+n)}. \tag{3}$$

(3) may be rewritten as a linear relation between the observable quantities (np) and p:

$$np=-mp+K'm. \tag{4}$$

By comparing different multiplicities of superinfection, K' may be eliminated

$$\frac{p}{p'}=\frac{m+n'}{m+n}. \tag{5}$$

To determine the pool size in induced defective lysogens, we studied recombination between the clear-plaque marker, $v1$, and the defect $i1$ or $i2$ when the superinfecting phage was $v1\ i1^+ i2^+$ and the carried phage $v1^+ i1\ i2^+$ or $v1^+ i1^+ i2$; in such an experiment the only viable recombinant is $v1^+ i1^+ i2^+$. The complementary recombinant $v1\ i1$ or $v1\ i2$ is not scorable. To determine the pool size in induced healthy lysogens, we studied recombination between the markers $v1$ and h when the superinfecting phage was $v1\ h$ and the carried phage was $v1^{+}\,h^{+}$. Scoring only one recombinant class $v1^{+}\,h$ among the total h phage in this case is analogous to the method of estimating the pool size in defective lysogens.

Validity of the method. The linearity of the relation (4) between (np) and p has been confirmed over a wide range of times and multiplicities of superinfection. A typical set of data is given in Table 1 and plotted as a test of linearity in Fig. 2. The straight lines derived from a number of other superinfections are plotted in Figs. 3 and 4.

Table 1. *Calculation of the values* (np) *and* p *of text relation* (4) *using experimental data from superinfection of* Escherichia coli C112 $(\lambda\ i2)$ *with phage* $\lambda\ v1\ i2^{+}h$ *15 min. after induction with u.v. light*

The values (np) and p are plotted in Fig. 2.

n*	$v1^{+}\,i2^{+}$	$v1\,i2^{+}$	p	np
1·6	224	2763	0·075	0·110
4·7	221	3738	0·055	0·258
10·0	115	3184	0·035	0·350
21·2	140	6136	0·021	0·445

* In order to allow for the Poisson distribution of the infecting particles, the values of n are calculated from the observed average multiplicity of infection, x, by the formula $n = x/(1 - e^{-x})$.

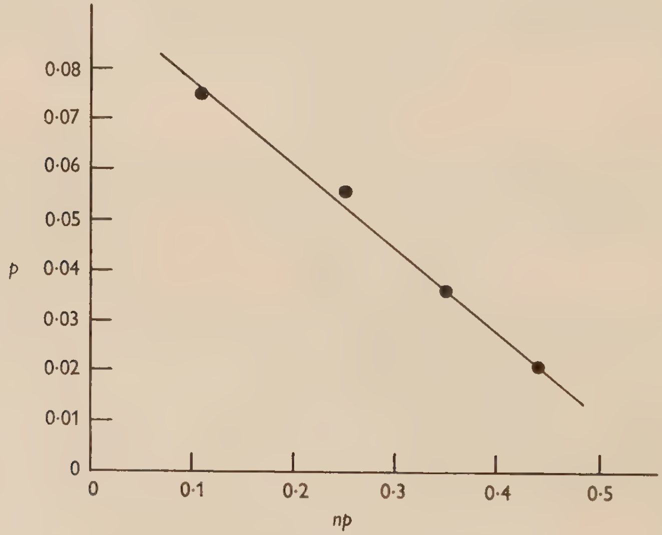

Fig. 2. Test of the linearity of relation (4) in the text between (np) and p. Data obtained from superinfection of *Escherichia coli* C112$(\lambda\ i2)$ with $v1\ i2^{+}h$ at 15 min. after u.v. induction. The data and calculations are recorded in Table 1. This line is also line *B* of Fig. 4.

Such straight lines as that of Fig. 2 have confirmed the validity of the assumptions concerning random mating and absence of selection upon which our estimates of pool size are based. They show that very high multiplicities of superinfecting phage can enter the induced cell and take part in recombination, in contrast to the situation in multiple infections of sensitive cells (Wollman & Jacob, 1954). Further, any selection for or against the superinfecting phage during multiplication should be a function of the extent of that multiplication and so, inversely, of the multiplicity of superinfection: hence it would give rise to curvature of the lines which is not observed. Loss of a constant appreciable fraction of the superinfecting phage, for example during penetration of the induced cell, is not excluded by the linearity and could give rise to a constant factor of error in the estimates of pool size. However, this is unlikely, in view of the good agreement of the pool size estimated shortly after induction (see Fig. 2 and below) with the number of prophages per cell estimated by other methods.

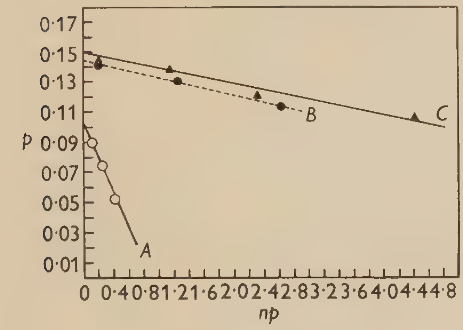

Fig. 3

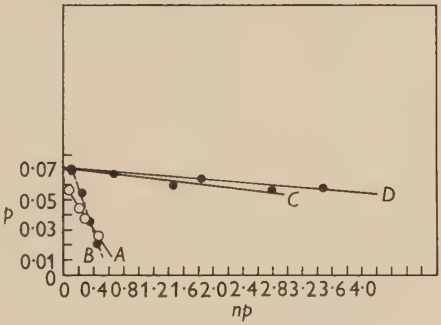

Fig. 4

Fig. 3. Determination of pool size (*m*) by linear plots of relation (4) of text.
A: *Escherichia coli* C112 ($\lambda\,s$) superinfected at 15 min. after induction. Pool size = 8·8.
B: *E. coli* C112 ($\lambda\,s$) superinfected at 40 min. after induction. Pool size = 95·4.
C: *E coli* C112 ($\lambda\,s$) superinfected at 45 min. after induction. Pool size = 95·2.

Fig. 4. Determination of pool size (*m*) by linear plots of relation (4) of text.
A: *Escherichia coli* C112 ($\lambda\,i1$) superinfected 43 min. after induction. Pool size = 12·4.
B: *E. coli* C112 ($\lambda\,i2$) superinfected 15 min. after induction. Pool size = 5·9.
C and *D*: *E. coli* C112 ($\lambda\,i2$) superinfected 45 min. after induction. Average pool size = 201.

Measurements of pool size as a function of time

We have used the recombinational analysis just described to follow the size of the genetic pools as a function of time in the defective lysogens *Escherichia coli* C112 ($\lambda\,i1$), C112 ($\lambda\,i2$), and the healthy lysogen *E. coli* C112 ($\lambda\,s$). The results are shown in Figs. 5 and 6. The three strains behave differently and will be discussed in succession.

Healthy lysogen (Fig. 5, *A*). During the first 15 min. after infection the number of recombinable particles remained nearly constant, but by 35 min. it had increased from 8 to 20. By 40 min. the pool size had increased to 95 particles and there it remained up to 45 min. This increase with time is reflected in the difference between the slope of line *A* and lines *B* and *C* of Fig. 3. The observations of Weigle & Delbrück (1951), that the first intracellular mature phage particles appeared between 40 and 45 min. after induction have been shown to apply to lysogenic derivatives of *Escherichia coli* of C112 under the conditions of our experiments (Appleyard, unpublished). We therefore attribute the levelling off of the pool size to the onset of maturation.

Defective lysogen Escherichia coli *C*112 ($\lambda\ i2$) (Fig. 5, *B*). The number of recombinable particles in induced *E. coli* C112 ($\lambda\ i2$) at first remained constant and then rose steeply as in *E. coli* C112 ($\lambda\ s$). However, instead of reaching a plateau it continued to rise to about 201 particles, twice the value in the healthy strain, and we were not able to detect any levelling off at the latest times at which we were able to make measurements. The rate of multiplication of phage $\lambda\ i2$ appeared to be about the same as that of phage $\lambda\ s$ (compare Fig. 5, *A B* with 5, *C*). These results suggest that the maturation of $\lambda\ i2$ is impaired whereas its vegetative multiplication is normal.

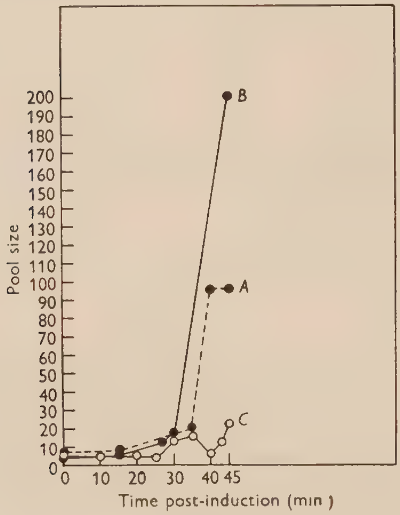

Fig. 5

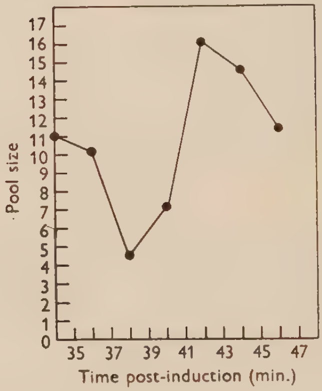

Fig. 6

Fig. 5. Variation of the pool size with time following induction. *A*: *Escherichia coli* C112 ($\lambda\ s$). *B*: *E. coli* C112 ($\lambda\ i2$). *C*: *E. coli* C112 ($\lambda\ i1$).

Fig. 6. Variation of the pool size with time following induction of *Escherichia coli* C112 ($\lambda\ i1$).

Defective lysogen Escherichia coli *C*112 ($\lambda\ i1$) (Figs. 5, *C* and 6 and Table 2). There were marked differences between the events in *E. coli* C112 ($\lambda\ i1$) and those in *E. coli* C112 ($\lambda\ i2$). The first multiplication of phage $\lambda\ i1$ took place between 25 and 30 min. after induction but was followed by a decline in numbers between 30 and 40 min. Two other experiments were performed to confirm this picture. In the second experiment the value of m was 14·6 at 30 min. and 16·3 at 35 min.; by 40 min. this number had declined to 8·8 and at 45 min. m had increased to 21·1. The results of these two experiments have been combined and presented in Fig. 5, *C* and Table 2.

Further analysis of the events described in the preceding paragraph was carried out. Samples of an induced culture of *Escherichia coli* C112 ($\lambda\ i1$)

were superinfected at 2 min. intervals from 34 to 46 min. From the data presented in Fig. 6 it may be seen that as in the first two experiments the value of m between 34 and 36 min. was constant, or nearly so, at 10. However, by 38 min. m had decreased to 4·5, being followed by the usual rise, which in this case began between 40 and 42 min.

Table 2. *Calculation of the pool size* (m) *in cells of* Escherichia coli C112 ($\lambda\, i1$) *superinfected with* $\lambda\, v1\, i1^{+}h$ *at various times post-induction*

Time post-induction (min.)	n	$v1^{+}\, i1^{+}$	$v1\, i1^{+}$	p	m
0	1·6	143	1547	0·085	5·5
	10·0	84	2020	0·039	
10	1·6	342	4341	0·073	4·3
	10·0	106	3340	0·030	
20	1·6	110	1218	0·083	4·8
	10·0	182	4740	0·036	
25	1·6	156	1802	0·079	3·8
	10·0	55	1379	0·031	
30	1·6	203	3146	0·061	12·0
	10·0	206	4968	0·038	
	1·6	157	1662	0·086	15·2
	10·0	92	1502	0·057	
	1·6	84	1062	0·073	14·6
	5·2	115	1791	0·060	
35	1·6	145	1737	0·077	16·3
	7·0	302	4683	0·060	
40	1·6	228	2605	0·080	5·5
	10·0	46	1195	0·037	
	1·6	166	1746	0·086	8·8
	7·0	123	2090	0·055	
43	1·4	91	1520	0·056	12·4
	5·0	151	3270	0·044	
	7·6	61	1533	0·038	
	18·5	66	2586	0·026	
45	1·6	425	6734	0·059	24·7
	10·0	168	3462	0·046	
	1·6	208	3335	0·058	21·1
	8·0	50	1057	0·045	

The difference between the pool sizes at 43–45 min. after induction of *Escherichia coli* C112 ($\lambda\, i1$) and *E. coli* C112 ($\lambda\, i2$) is reflected in the difference between the slope of line *A* and those of lines *C* and *D* of Fig. 4. It appears that phage $\lambda\, i1$ can undergo only limited multiplication in the vegetative state.

DISCUSSION

Measurements of pool size and u.v.-stimulated liberation of healthy phage show that defects $i1$ and $i2$ bring about different physiological changes following induction. In particular, estimates of genetic pool size show that phage $\lambda\ i1$, unlike phage $\lambda\ i2$, experiences some difficulty in vegetative multiplication as was suggested by the curves of u.v.-stimulated reversions (Fig. 1, A). Comparison of the pool formed by each defective phage with that formed by healthy phage (Fig. 5) permits a more detailed interpretation of the nature of both defects.

The nature of defect $i2$. The characteristic difference between phage $\lambda\ i2$ and phage $\lambda\ s$ is the failure of the number of recombinable particles in the case of the induced $i2$ lysogen to stop rising and reach a plateau. Although the time at which the rapid increase in recombinable particles took place in *Escherichia coli* C112 ($\lambda\ i2$) was 5 min. earlier than that in *E. coli* C112 ($\lambda\ s$), this is more likely to be correlated with later lysis of induced *E. coli* C112 ($\lambda\ s$), than to be a characteristic of phage $\lambda\ i2$.

Weigle & Delbrück (1951) observed that the first mature infectious phages appeared in cells of induced healthy *Escherichia coli* K12 (λ) between 40 and 45 min. after induction. This observation has been confirmed for our strains (Appleyard, unpublished). If the attainment of a constant pool size in the induced normal lysogenic organism, as in coliphage T2- or T4-infected organisms, is due to the intervention of maturation, then we can describe the $i2$ defect as a failure to undergo the first step of maturation, i.e. removal from the vegetative pool.

How much of the machinery of maturation can be set up by phage $\lambda\ i2$ remains unknown. However, Dr E. Kellenberger (personal communication) observed that empty phage heads and tails both appeared in lysates after induction of the strain C33 ($\lambda\ i2$). Whitfield, Appleyard & Baird (unpublished) prepared doubly lysogenic strains of *Escherichia coli* C112 which carried both phage $\lambda\ i2$ and a host range (h) mutant of normal phage λ. Among the phage in lysates from such u.v.-induced strains, they observed healthy phage which were genotypically h but which possessed h^+ coats derived from the defective phage. Possession of an h coat permits the individual phage to absorb on to the host range indicator strain *E. coli* CR63 while a phage particle with an h^+ coat cannot absorb on to this strain (Appleyard *et al.* 1956). Therefore, it seems that phage $\lambda\ i2$ can initiate construction of and at least in part control the specificity of the morphological components required for maturation.

Another defective λ phage was studied by Jacob & Wollman (1956). They concluded that their defect prevents maturation of vegetative λ phage. Since these authors were unable to demonstrate the presence of mature defective phage even after superinfection with healthy phage, they suggested that each defective vegetative unit interferes with its own maturation. However, in the presence of healthy phage, $\lambda\ i2$ can be matured (Appleyard, 1956). We therefore conclude that it belongs to a different class of maturation defects.

The nature of $i1$. In the prophage state, phage $\lambda\ i1$ is stable, for it can be transmitted through many cell generations without loss. In induced and spontaneous lysates from the double lysogenic strain *Escherichia coli* C112 ($\lambda\ i1$, λ C1h), the major part of the progeny population is genotypically h but phenotypically h^+. It would appear from this that phage $\lambda\ i1$, as is the case with phage $\lambda\, i2$, can direct the synthesis of those elements which confer upon the mature virus its host range properties (Whitfield, Appleyard & Baird, unpublished).

In the vegetative state, phage $\lambda\, i1$ can multiply, since two successive bursts of multiplication are observed to follow induction. However, these two events are separated by an interval of decrease in the number of vegetative particles. Lambda-$i1$ phage is therefore unstable in the vegetative state. The present experiments do not enable us to distinguish clearly between a genuine physico-chemical instability of vegetative phage $\lambda\, i1$ and a cessation of multiplication coupled with some hypothetical normal process of removal from the vegetative pool. However, removal could not in this case be through a normal maturation process, as it begins too soon.

Some support for believing vegetative phage $\lambda\, i1$ to be unstable is provided by the observations of Appleyard & Whitfield (1956). They observed that superinfection immediately following u.v. irradiation of cultures of *Escherichia coli* C112 ($\lambda\, i2$) with phage $\lambda\, i1$ resulted in the appearance of no healthy recombinant phage-yielding organisms. Superinfection at later times produced increasing numbers of normal phage yielders. In the opposite situation, with phage $\lambda\, i2$ and *E. coli* C112 ($\lambda\, i1$), superinfection immediately following u.v. irradiation resulted in the appearance of nearly the maximum number of normal phage-yielders possible under the conditions of the experiment. These observations suggest that phage $\lambda\, i1$, unable to maintain its integrity in the host cytoplasm, would have to enter the host at a later time in order to be present in an intact state to participate in recombination with phage $\lambda\, i2$ when that phage begins its vegetative multiplication.

Analyses of the deoxyribonucleic acid content of *Escherichia coli* C112 ($\lambda\, i1$) cultures at various times after u.v.-induction indicate that the DNA content remains constant during the first 30 min. after induction but shortly thereafter increases linearly at the same rate as that in induced normal lysogenic cultures of *E. coli* C112 ($\lambda\, s$) (Whitfield, unpublished). It should be pointed out that the time of resumption of DNA synthesis following induction of these strains correlates well with the time at which the values of m begin to rise and also with the time at which the frequency of u.v.-stimulated reversions sharply increases in *E. coli* C112 ($\lambda\, i2$). These biochemical studies, to be published later, were carried out under the same conditions as the pool-size estimations.

If the instability of phage $\lambda\, i1$ be due to extensive fragmentation resulting from the operation of some factor such as deoxyribonuclease, then large amounts of DNA fragments would appear in the acid-soluble nucleotide fractions from 30 min. to lysis of the culture. This was not observed. However, limited fragmentation of the vegetative particles remains a possibility.

During the decline the number of recombinable particles decreases not to zero, but to approximately the number preceding the first burst of multiplication; thus there seems to be a small persisting number of stable particles. We suggest that this persistent fraction represents the original prophages of the cell which by continued association with the bacterial nuclear material retain their stability. This hypothesis implies that in the process of induction, prophage is not itself physically converted to vegetative phage, for all the intracellular particles would then be unstable, but makes at least one vegetative replica which initiates vegetative multiplication. In the case of the unstable phage $\lambda\, i1$, because the picture is not obliterated by large numbers of vegetative phage units, it can be seen to initiate two such attempts at vegetative multiplication, separated from each other and from the moment of induction by very approximately the division time of the un-irradiated lysogenic organisms.

The nature of u.v. radiation induction. We therefore propose the following hypothesis concerning the mechanism of u.v. radiation induction. The effect of the radiation is to modify the cell's nuclear apparatus in such a way that replicas formed by the prophage at its subsequent divisions become vegetative phage. It is of interest to relate this hypothesis in detail to a number of established facts. The work of Lwoff and his colleagues (Lwoff, 1953*b*) suggests the intervention of physiological processes in the induction process. Direct absorption of radiation energy by prophage is not needed to accomplish induction. We suggest that the effect of the radiation upon the nuclear apparatus is somewhat analogous to the mitotic delay well known as an effect of radiation upon the cells of higher organisms. DNA synthesis in both lysogenic and non-lysogenic microbial cells is inhibited by small doses of u.v. radiation (Siminovitch & Rapkine, 1951; Kelner, 1953; Barner & Cohen, 1956; Whitfield, unpublished), although various intermediates of DNA synthesis including deoxyribonucleotides appear as accumulated products in the medium (Kanazir, 1954; Kanazir & Errera, 1954). Such changes would prevent the multiplication of the bacterial nuclear apparatus. If the prophage DNA were not similarly affected, the fresh replica would find itself in a predominantly cytoplasmic milieu. The availability of accumulated DNA products would permit it to multiply as an autonomous unit. The unbalanced condition of the cell would thus greatly favour vegetative multiplication. Similar hypotheses have been proposed by Jacob & Stent (cited by Lwoff, 1953*a*).

Morse, Lederberg & Lederberg (1956) observed that in *Escherichia coli* transduction of *gal* markers by λ phage was effective only when the phage was obtained by induction of a lysogenic strain, and is not observed when the phage issued from a lytic infection. Their findings are most naturally explained if vegetative λ phage does not become attached to galactose loci but after induction the linkage of some units to *gal* markers which they initially had as prophages persists, as does the associated stability in our experiments.

The number of prophages/organism. Bertani (1953) and Jacob (1954) were able to obtain approximate calculations of the number of prophages/lysogenic bacterium. Both workers (Bertani with lysogenic *Shigella dysenteriae* and Jacob with lysogenic *Pseudomonas aeruginosa*) concluded that the number of prophages/bacterium was close to the number of nuclei. The present studies lead to the same conclusion. The number of prophages can be inferred from the number of recombinable particles at zero minutes after induction. This number ranges from 4·6 to 6·0 with an average of about 5 and so closely approximates the average number of nuclei/cell in the logarithmic phase of growth (Whitfield, unpublished).

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REFERENCES

APPLEYARD, R. K. (1954). Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. *Genetics*, **39**, 440.

APPLEYARD, R. K. (1956). The transfer of defective *lambda* lysogeny between strains of *Escherichia coli*. *J. gen. Microbiol.* **14**, 573.

APPLEYARD, R. K., MCGREGOR, J. F. & BAIRD, K. M. (1956). Mutation to extended host range and the occurrence of phenotypic mixing in the temperate coliphage *lambda*. *Virology*, **2**, 565.

APPLEYARD, R. K. & WHITFIELD, J. F. (1956). Hereditary defects of temperate bacteriophage. *Nature, Lond.*, **178**, 1235.

BARNER, H. D. & COHEN, S. S. (1956). The relation of growth to the lethal damage induced by ultraviolet irradiation in *Escherichia coli*. *J. Bact.* **71**, 149.

BERTANI, G. (1953). Lysogenic versus lytic cycle of phage multiplication. *Cold Spr. Harb. Symp. quant. Biol.* **18**, 65.

JACOB, F. (1954). *Les bactéries lysogènes et la notion de provirus*. Paris: Masson et Cie.

JACOB, F. & WOLLMAN, E. L. (1954). Étude génétique d'un bactériophage tempéré d'*Escherichia coli*. I. Le système génétique du bactériophage λ. *Ann. Inst. Pasteur*, **87**, 653.

JACOB, F. & WOLLMAN, E. L. (1955). Étude génétique d'un bactériophage tempéré d'*Escherichia coli*. III. Effect du rayonnement ultraviolet sur la recombinaison génétique. *Ann. Inst. Pasteur*, **88**, 724.

JACOB, F. & WOLLMAN, E. L. (1956). Recherches sur les bactéries lysogènes defectives. I. Déterminisme génétique de la morphogenèse chez un bactériophage tempéré. *Ann. Inst. Pasteur*, **90**, 282.

KANAZIR, D. (1954). Accumulation de l'acide thymidylique chez *E. coli*. après irradiation UV. *Biochim. biophys. Acta*, **13**, 587.

KANAZIR, D. & ERRERA, M. (1954). Métabolisme des acides nucléiques chez *E. coli* B après irradiation ultraviolette. *Biochim. biophys. Acta*, **41**, 62.

KELNER, A. (1953). Growth, respiration, and nucleic acid synthesis in ultraviolet-irradiated and in photoreactivated *Escherichia coli*. *J. Bact.* **65**, 252.

LEVINTHAL, C. & VISCONTI, N. (1953). Growth and recombination in bacterial viruses. *Genetics*, **38**, 500.

LWOFF, A. (1953*a*). L'induction. *Ann. Inst. Pasteur*, **84**, 225.

LWOFF, A. (1953*b*). Lysogeny. *Bact. Rev.* **17**, 269.

MORSE, M. L., LEDERBERG, E. M. & LEDERBERG, J. (1956). Transduction in *Escherichia coli* K12. *Genetics*, **41**, 142.

SIMINOVITCH, L. & RAPKINE, S. (1951). Modifications biochimiques au cours du développement des bactériophages chez une bactérie lysogène. *C.R. Acad. Sci., Paris*, **232**, 1603.

VISCONTI, N. & DELBRÜCK, M. (1953). The mechanism of genetic recombination in phage. *Genetics*, **38**, 5.

VISCONTI, N. & GAREN, A. (1953). Unity of the vegetative pool in phage-infected bacteria. *Proc. nat. Acad. Sci., Wash.* **39**, 620.

WEIGLE, J. J. & DELBRÜCK, M. (1951). Mutual exclusion between an infecting phage and a carried phage. *J. Bact.* **62**, 301.

WOLLMAN, E. L. (1953). Sur le déterminisme génétique de la lysogénie. *Ann. Inst. Pasteur*, **84**, 281.

WOLLMAN, E. L. & JACOB, F. (1954). Étude génétique d'un bactériophage tempéré d'*Escherichia coli.* II. Mécanisme de la recombinaison génétique. *Ann. Inst. Pasteur*, **87**, 674.

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Goodwin, T. W. (1957). *J. gen. Microbiol.* **17**, 467–473

The Nature and Distribution of Carotenoids in some Blue-Green Algae

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SUMMARY: Seven blue-green algae, *Anabaena cylindrica, A. variabilis, Coccochloris elabens, Cylindrospermum* sp., *Mastigocladus laminosus, Microcoleus vaginatus* and *Nostoc* sp., synthesize the same three major carotenoids: β-carotene, echinenone and myxoxanthophyll; zeaxanthin is present in small amounts. Lutein could not be detected. β-Carotene represents between 30 and 60% of the total carotenoids present. The carotenoid distribution in blue-green algae and other photosynthetic organisms is discussed.

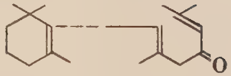
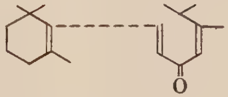
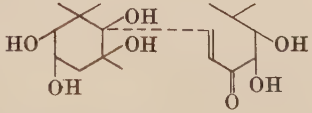
From the viewpoint of carotenoid biochemistry the small number of blue-green algae (Myxophyceae or Cyanophyceae) already studied (*Calothrix scopulorum, Oscillatoria rubescens, Rivularia* spp. and *Aphanizomenon flos-aquae*) show a number of interesting aspects (Goodwin, 1952, 1957). These algae are characterized by: (*a*) the absence (or occurrence only in minute traces) of lutein, the major xanthophyll of the chloroplasts of higher plants; (*b*) the occurrence of a monoketocarotenoid never found in other algae or higher plants (one such compound has recently been isolated from a photosynthetic bacterium (Goodwin, Land & Sissins, 1956)); (*c*) the occurrence of characteristic polyhydroxycarotenoids not yet observed in other photosynthetic tissues.

The present investigation was undertaken to see whether this distribution was more general amongst the Cyanophyceae, to resolve some doubtful points concerning the identity of some pigments already reported present in blue-green algae, and to determine the quantitative distribution of the pigments. The situation prior to the present investigation is summarized in Table 1, which shows that the properties of myxoxanthin and aphanin are very similar and it has been claimed (Goodwin & Taha, 1951) that they are identical with echinenone (4-keto-β-carotene) (Ganguly, Krinsky & Pinckard, 1956; Goodwin, 1956*a*). Myxoxanthophyll and aphanizophyll are also very similar.

METHODS

Cultures. The cultures, all obtained from Dr G. E. Fogg (Department of Botany, University College, London), were *Anabaena cylindrica* Lemm, *A. variabilis* Kützing (Kratz & Myers, 1955), *Coccochloris elabens* (Bréb) Dr. & Daily, *Cylindrospermum* sp. (isolated by Dr G. E. Fogg), *Mastigocladus laminosus* Cohn, *Microcoleus vaginatus* and *Nostoc* sp. (an unidentified species obtained by Dr Fogg from Dr R. A. Lewin, Woods Hole). With the exception of *Anabaena cylindrica* and *A. variabilis*, they were examined as received from

Table 1. *Specific carotenoid pigments already reported in blue-green algae**

Pigment	Source	M.p. (°)	Absorption spectrum maximum (mμ.)			Possible structure	Reference
			CS_2	$CHCl_3$	Light petroleum, CH_3OH, or C_2H_5OH		
Aphanin†	*Aphanizomenon flos-aquae*	—	494	474	460		Tischer (1938, 1939)
Myxoxanthin†	*Rivularia nitida* *Oscillatoria rubescens*	168–169	488	473	465		Heilbron & Lythgoe (1936)
Flavacin‡	*A. flos-aquae*	155	490 457 424	— —	458 428	Unknown	Tischer (1938, 1939)
Aphanizophyll	*A. flos-aquae*	172–177	—	523 487·5 457	CH_3OH 507 475 444	Unknown	Tischer (1938, 1939)
Oscillaxanthin	*O. rubescens*	—	568 528 494	—	531 496 464	Acidic in nature	Karrer & Rutschmann (1944)
Aphanicin	*A. flos-aquae*	190	533 494	504	491·5 457	(?) A dicarotenoid containing two aphanin molecules joined by an oxygen bridge	Tischer (1938)
Myxoxanthophyll	*O. rubescens*	182	—	518 484 454	C_2H_5OH 503 471 445		Karrer & Rutschmann (1944)

* β-Carotene is present in all algae examined.

† These are probably identical with echinenone (4-keto-β-carotene) (Goodwin & Taha, 1951; and this paper).

‡ May be identical with mutatochrome (Karrer & Jucker, 1950).

Dr Fogg. Cultures of *A. cylindrica* were grown on the medium described by Allen & Arnon (1955); we are grateful to Dr J. Glover of this department for these cultures.

Extraction of pigments. Ethanol was added to the organisms and the mixture immersed in a boiling water-bath for 2 min. with continuous stirring; it was then filtered through a sintered glass filter (G4), the extraction of the residue was repeated until all pigment was removed. An equal volume of diethylether was added to the combined ethanol extracts, followed by the addition of water dropwise until two layers were formed. The ethereal epiphase, containing all the pigments, was washed free from ethanol with water, and the solvent removed. The residue was then saponified (Goodwin, 1955*a*) to destroy the chlorophylls and to remove lipids which might have interfered with the chromatography. It was found better to saponify at this stage rather than earlier, as advocated for Chlorella (Goodwin, 1954). The unsaponifiable residue was dissolved in a little ether and then 10 ml. light petroleum (b.p. 40–60°) added. The solution was then ready for chromatography. The trace of ether is necessary to dissolve myxoxanthophyll which is insoluble in light petroleum.

Separation, characterization and identification of pigments. The pigments were separated chromatographically on columns of $CaCO_3$ (analytical grade), icing (confectioner's) sugar (Goodwin, 1955*a*) or alumina deactivated with methanol (Goodwin & Srisukh, 1949). They were characterized by their chromatographic behaviour, their absorption spectra in a number of solvents and by special tests such as reduction of a carbonyl grouping with $LiAlH_4$ (Goodwin, 1955*a*, 1956*a*) or treatment with chloroform saturated with hydrogen chloride to reveal an allylic hydroxyl group (Karrer & Leumann, 1951).

Pigments were identified by these tests, combined whenever possible with mixed chromatography on two adsorbents with an authentic sample of the suspected pigment as marker (Goodwin, 1955*a*).

Quantitative determination of constituent pigments. The amounts of the pigments present were determined by measuring the E ($\lambda_{\text{max.}}$) value of each extract and comparing it with the $E^{1\%}_{1\,\text{cm.}}$ value of the authentic pigment (Karrer & Jucker, 1950; Goodwin, 1952, 1955*b*). Where no such value was available it was assumed to be that of β-carotene, namely, 2500 (Goodwin, 1955*a*).

RESULTS

Nature of the pigments

All the algae were found to contain the same pigments so that each separation need not be detailed. A typical separation is recorded in Table 2. The first three pigments were identified as β-carotene, echinenone and zeaxanthin by their absorption spectra and by mixed chromatography with authentic specimens. The identity of echinenone was further confirmed by reduction with $LiAlH_4$ to 4-hydroxy-β-carotene (isocryptoxanthin; Zechmeister & Wallcave, 1953), which on treatment with $CHCl_3+HCl$ loses water to give dehydro-β-carotene which was identified by its conversion to β-carotene by treatment with BF_3 (Zechmeister & Wallcave, 1953; Goodwin, 1956*a*). As echinenone is present in all the seven algae examined it would appear that the previous claim that echinenone, myxoxanthin and aphanin are identical (Goodwin & Taha, 1951) is justified.

Table 2. *A typical chromatographic separation of the carotenoids of a blue-green alga*

Adsorbent confectioner's sugar; developing solvent light petroleum containing varying amounts of acetone; zones in order of increasing adsorptive affinity.

Zone no.	Colour	Percentage acetone (v/v) in light petroleum required for elution	Absorption maxima (mμ.) in light petroleum	Identification
1	Yellow	0	426, 448, 474	β-Carotene
2	Brownish red	0·5	452	Echinenone
3	Yellowish brown	2·0	425, 448, 474	Zeaxanthin
4	Bright red	Removed only with ethanol	450, 475, 506*	Myxoxanthophyll

* Measured in ethanol.

The most strongly adsorbed pigment (4, Table 2) is undoubtedly closely related to, if not identical with, myxoxanthophyll (Heilbron & Lythgoe, 1936) and aphanizophyll (Tischer, 1938, 1939). Heilbron (1942) considered that these two pigments were identical and the fact that the corresponding pigments from all our cultures are also identical would tend to confirm this conclusion; for convenience in this paper we shall use the name myxoxanthophyll. The structure suggested for myxoxanthophyll by Karrer & Rutschmann (1944: see Table 1) is probably incorrect in detail because we find that although it reacts with $LiAlH_4$, indicating the presence of a carbonyl grouping, the reduced pigment has an absorption spectrum very like that of the original pigment. This indicates the presence of a carbonyl grouping isolated from the main polyene chain; reduction of the carbonyl in Karrer & Rutschmann's formula would result in a very marked shift to lower wavelengths (Goodwin *et al.* 1956). Furthermore, a carotenoid containing a conjugated carbonyl group would almost certainly have a much less well-defined spectrum than has myxoxanthophyll (Goodwin, 1952, 1955*a*).

The absence of lutein, commented on by previous workers, has been confirmed in all cases. Its occasional presence in traces as recorded previously might have been due to the fact that the investigators were using natural mass cultures and not pure cultures. Flavacin and oscillaxanthin, occasionally noted previously, were not detected in any of the algae examined in this survey.

Quantitative data

The values given in Table 3 are for cultures at least 2 weeks old; minor quantitative differences might be observed in cultures of different ages. It will be seen that in all cases except *Microcoleus vaginatus*, which is exceptionally low in echinenone, zeaxanthin was always the minor component and that the relative amounts of the other components varied considerably from one alga to another. Except in one case (*Nostoc* sp.), the proportion of β-carotene was $>30\%$ of the total carotenoids and was generally *c.* 50%; this high proportion of β-carotene is very characteristic (see Discussion). If the carotene/xanthophyll ratios for these algae are calculated, they agree reasonably well with those given by Handke (1954).

Table 3. *The quantitative distribution of carotenoids in seven blue-green algae*

	Pigment			
	β-Carotene	Echinenone	Zeaxanthin	Myxoxanthophyll
	(% total pigment)			
Anabaena cylindrica	54	19	Trace	17
A. variabilis	34	28	4	33
Coccochloris elabens	42	16	Trace	42
Cylindrospermum sp.	50	25	Trace	25
Mastigocladus (*a*)	42	39	8	11
(*b*)	42	40	9	9
Microcoleus vaginatus	63	6	9	22
Nostoc sp.	28	53	Trace	19

DISCUSSION

The present data, together with those previously reported, indicate that the blue-green algae are unique amongst photosynthetic organisms in their carotenoid distribution. Photosynthetic organisms may be divided into six groups according to the nature of their constituent carotenoids: (1) all green tissues of higher plants and the algal classes Chlorophyceae, Xanthophyceae, Euglenineae and Rhodophyceae*; these contain β-carotene, lutein and neoxanthin as their major pigments (Goodwin, 1957); (2) the brown algae (Phaeophyceae) and diatoms (Bacillariophyceae) where fucoxanthin replaces lutein and neoxanthin (Goodwin, 1957); (3) the blue-green algae (Cyanophyceae), with echinenone and myxoxanthophyll replacing lutein and neoxanthin; (4) the Dinophyceae (Peridinieae) in which a xanthophyll of unknown structure, peridinin, replaces lutein; (5) purple sulphur and non-sulphur photosynthetic bacteria which are characterized by the absence of any 'normal' plastid pigments and produce open-chain carotenoids often containing, very characteristically, methoxyl groups (Goodwin, 1955*b*, 1956*b*); (6) the green photosynthetic bacteria; these accumulate mainly γ-carotene (Goodwin & Land, 1956).

The following points should also be noted. The blue-green algae are the only photosynthetic tissues in which often about one-half the total carotenoid is β-carotene (25% is a normal value for higher plants and 10% for green algae). The unique carotenoid distribution of the blue-green algae tends to support the general view that they have existed apart from the main line of evolution from a very early age (Fogg, 1956). In the plant world echinenone is, as far as is known, specific to the blue-green algae, being found otherwise only in the animal kingdom, and it has been suggested that the occurrence of echinenone in lake deposits might be used to determine the growth of blue-green algae in the past (Vallentyne, 1954). In spite of sharing with the blue-green algae the unique ability to synthesize bilichromoproteins (phycobilins), the red algae show no individuality in their carotenoid make-up which is almost identical with that of the higher plants (Goodwin, 1957). Although qualitative carotenoid analysis could be used with confidence to help identify an alga as a member of the Cyanophyceae, considerably more quantitative work is required before the relative distribution of the constituent pigments could be taken to indicate one particular genus.

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* Nothing is yet known of the carotenoids in the Cryptophyceae and Chloromonadineae.

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REFERENCES

Allen, M. B. & Arnon, D. I. (1955). Growth and nitrogen fixation in *Anabaena cylindrica*. *Plant Physiol.* **30**, 366.

Fogg, G. E. (1956). The comparative physiology and biochemistry of the blue-green algae. *Bact. Rev.* **20**, 148.

Ganguly, J., Krinsky, N. I. & Pinckard, J. H. (1956). Isolation and nature of echinenone, a provitamin A. *Arch. Biochem. Biophys.* **60**, 345.

Goodwin, T. W. (1952). *The Comparative Biochemistry of the Carotenoids*. London: Chapman and Hall.

Goodwin, T. W. (1954). Some observations on carotenoid synthesis by the alga *Chlorella vulgaris*. *Experientia*, **10**, 213.

Goodwin, T. W. (1955*a*). Carotenoids. In *Modern Methods of Plant Analysis*. Ed. Paech, K. & Tracey, M. V. Heidelberg: Springer.

Goodwin, T. W. (1955*b*). Carotenoids. *Annu. Rev. Biochem.* **24**, 497.

Goodwin, T. W. (1956*a*). Studies in carotenogenesis. 22. The structure of echinenone. *Biochem. J.* **63**, 481.

Goodwin, T. W. (1956*b*). The nature, biosynthesis and function of the carotenoids in the photosynthetic bacteria. *Soc. Biol. Chemists, India, Souvenir*, p. 271.

Goodwin, T. W. (1957). Nature, biosynthesis and function of carotenoids in photosynthetic tissues. In *Handbook of Plant Physiology*, **5**. Heidelberg: Springer.

Goodwin, T. W. & Land, D. G. (1956). Studies in carotenogenesis. 20. Carotenoids of some species of *Chlorobium*. *Biochem. J.* **62**, 553.

Goodwin, T. W., Land, D. G. & Sissins, M. E. (1956). The nature of the carotenoids in the photosynthetic bacterium *Rhodopseudomonas spheroides*. *Biochem. J.* **64**, 486.

Goodwin, T. W. & Srisukh, S. (1949). The biochemistry of locusts. 1. The carotenoids of the integument of the two locust species *Locusta migratoria migratoroides* R. & F. and *Schistocerca gregaria* Forsk. *Biochem. J.* **45**, 263.

Goodwin, T. W. & Taha, M. M. (1951). A study of the carotenoids echinenone and myxoxanthin with special reference to their probable identity. *Biochem. J.* **48**, 513.

Handke, H. H. (1954). Quantitative Untersuchungen über die alkohollöslichen Pigmente bei Cyanophyceen. *Wiss. Z. Martin Luther Universität Halle Wittenberg*, **4**, 89.

Heilbron, I. M. (1942). Some aspects of algal chemistry. *J. chem. Soc.* p. 59.
Heilbron, I. M. & Lythgoe, B. (1936). The carotenoid pigments of *Oscillatoria rubescens*. *J. chem. Soc.* p. 1376.
Karrer, P. & Jucker, E. (1950). *Carotenoids*. Translated Braude, E. A. London: Elsevier.
Karrer, P. & Leumann, E. (1951). Eschscholtzxanthin und Anhydroeschscholtzxanthin. *Helv. chim. Acta*, **34**, 445.
Karrer, P. & Rutschmann, J. (1944). Beitrag zu Kenntnis der Carotinoide aus *Oscillatoria rubescens*. *Helv. chim. Acta*, **27**, 1691.
Kratz, W. A. & Myers, J. (1955). Nutrition and growth of several blue-green algae. *Amer. J. Bot.* **42**, 282.
Tischer, J. (1938). Über die Polyenpigmente der Blaualge *Aphanizomen flos-aquae*. *Hoppe-Seyl. Z.* **251**, 109.
Tischer, J. (1939). Über die Polyenpigmente der Blaualge Aphanizomenon flos-aquae. II. *Hoppe-Seyl. Z.* **260**, 257.
Vallentyne, J. R. (1954). Biochemical limnology. *Science*, **119**, 605.
Zechmeister, L. & Wallcave, L. (1953). A study of *cis-trans* isomeric dehydro-β-carotenes. *J. Amer. chem. Soc.* **75**, 5341.

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The Action of Chemical Disinfectants on Bacteria in Droplets Compared with that in Large Volumes

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SUMMARY: Because of adsorption phenomena, copper sulphate solutions containing traces of protein are less toxic to bacteria in droplets than to similar bacteria in large volumes. With dilute phenol solutions the reverse was found. The quantitative results suggest that the non-flagellate organism studied moves with about 25 times the energy of Brownian motion.

Although much information is available about the killing of bacteria by different chemicals, almost all of it has been obtained from investigations that used volumes of suspensions of the order of 1 ml. or more. Little has been published about the rate of killing of bacteria suspended in droplets owing, for the most part, to the difficulty of adapting the standard viability counting methods for use with such small samples. However, the urea method of measuring bacterial viability (Valentine & Bradfield, 1954) does allow the % live organisms in small drops to be determined. In outline this method consists of incubating a drop of the suspension under investigation on the film of an electron microscope specimen support placed on a nutrient agar medium containing 3% (w/v) urea. The urea does not stop the growth of any live bacteria in the drop but it does inhibit their division and each develops into a long filamentous form. The dead bacteria remain unaltered in appearance. After about 3 hr. of incubation, the film is fixed in formalin vapour, washed and examined in the electron microscope. A determination of the ratio of long to normal-length organisms then gives a reliable estimate of the ratio of live to dead bacteria originally in the drop. In the experiments now described, this urea method has been used to measure the rate of killing of bacteria by dilute copper sulphate and phenol solutions in drops 0·6 mm. in diameter. Observation of bacteria in drops of this size with the light microscope showed that when the surface tension of the suspending fluid was about that of water, the bacteria arriving at the surface of the drop were held there by surface forces and unable to return to the interior. Within a few minutes, many of the bacteria in a small drop have gathered and remain on the surface and it is here that much of the interaction between them and any chemicals in the drop takes place. Adsorption phenomena may cause the chemical composition of this surface layer to differ from that of the interior and thus it is quite possible that the death-rate of bacteria in droplets containing toxic substances may be entirely different from that in larger volumes of the same suspension. This point is in fact demonstrated by the following experiments.

METHODS

Bacteria. A paracolon bacterium designated as D. 201 H (Fry & Greaves, 1951) kindly supplied by Dr R. M. Fry was used; it is deposited in the National Collection of Type Cultures (Colindale) as NCTC 9793. It was grown on nutrient agar slopes prepared from the Difco dried product. Suspensions were made by removing sufficient growth from 18 hr. cultures and rapidly suspending it in the solutions under investigation. These were made to contain about 10^7 bacteria/ml. of which, in all cases, 90 % or more were initially viable as measured by the urea method.

Formation of droplets. A drop of the suspension was taken up immediately in a platinum loop. It was held in the same tube just above the surface of the suspension from which it had been taken. The tube was closed and there was negligible evaporation of the drop in 30 min. which was the longest time involved in these experiments. By bending the platinum wire so that the loop was in a horizontal plane when removed from the liquid, the drop so formed was nearly a perfect sphere and had a diameter of 0·6 mm. After the desired length of time, the drop was transferred for a viability count on to the film of the specimen support. This had already been placed on the nutrient agar containing 3 % (w/v) urea in a Petri dish warmed to 37°. A second drop was taken at the same time from the bulk suspension and placed on a second film in the same dish. The two samples thus received identical treatment except that in one the bactericidal action took place in a droplet and in the other in a comparatively large volume. The amount of the decrease in the viability in each case will be referred to respectively as killing in the drop and in bulk suspension. It was found that with the weak solutions used the bactericidal action could be stopped immediately the drop was on the film by piercing a very small hole in this with a drawn-out fibre of glass so that the drop diffused into the agar.

Viability counts. Counts were made by the urea method in the manner previously described (Valentine & Bradfield, 1954). The results given are the mean counts made on three similar drops in each case. The errors quoted are the standard errors of this mean calculated as previously described from the total number of bacteria observed. No significant differences were found in the counts made on similar drops.

RESULTS

Disinfection with copper sulphate

The effect of various concentrations of copper sulphate on the bacteria suspended in 5 ml. volumes was first investigated. As is usual with metallic salts, the toxicity of the copper sulphate was found to be greatly decreased when broth or other material containing soluble protein was also present in solution. Even the small amount of broth carried over with the bacteria from the culture slope had an appreciable protective action. Thus there was markedly greater killing with organisms washed free from this by being shaken in distilled water and then centrifuged down, as compared with the killing of unwashed organisms. The results obtained are shown in Table 1.

Table 1. *Effect of various concentrations of copper sulphate on washed and unwashed organisms*

Concentration of $CuSO_4$ solution (M)	Bacteria killed in 30 min. Unwashed organisms (%)	Bacteria killed in 30 min. Washed organisms (%)
10^{-4}	100	100
3×10^{-5}	88	100
10^{-5}	60	95
10^{-6}	0	0

To study the effect of droplet suspension on the rate of disinfection, 10^{-5} M-copper sulphate solution was used. Suspensions of washed and unwashed organisms were prepared as before and this time viability counts were made on drops (0·6 mm. diameter) as well as on the bulk suspensions. Table 2 shows the % organisms killed in 30 min. under the various conditions. These results leave little doubt that the bactericidal action of the copper ions was markedly less in the drops than in bulk suspensions with the unwashed organisms, i.e. when a certain amount of soluble protein was also present. Many other similar experiments have always confirmed this point. The difference is less marked with washed organisms and becomes negligible when this washing has been repeated a number of times.

Table 2. *Effect of copper sulphate on organisms in droplets compared with the effect in larger volumes*

	Organisms killed in 30 min. in 10^{-5} M-$CuSO_4$ solution: In bulk (%)	Organisms killed in 30 min. in 10^{-5} M-$CuSO_4$ solution: In drop (%)
Washed organisms	94 ± 1	80 ± 2
Unwashed organisms	60 ± 2·5	10 ± 1·5

The rate of killing of unwashed organisms in drops and in bulk suspensions was then studied by determining the viability of samples at various times up to 20 min. Table 3 shows how the % organisms killed by 3×10^{-5} M-copper sulphate solution varied with time. After 10 min., the rate of killing in the bulk suspension was at least twice as great as in the drops and increased still further after this.

In order to confirm that the difference between survival in bulk suspensions and in the drops was a true surface effect, the result of adding a surface active agent was investigated. It was found that "Teepol X" even at high concentrations (1/10) had no toxic action on the bacteria. At the same time a 1/10,000 dilution decreased the surface tension of water from 72 to 40 dynes cm.$^{-1}$ and at this lowered surface tension the bacteria were seen to move freely from the surface back into the interior of the drop instead of being held by the surface forces. As expected, it was found that there was then no significant difference between the killing in bulk suspensions and in the drops. Table 4 shows the % of unwashed organisms killed after 15 min. in 3×10^{-5}M-copper sulphate, with and without the addition of Teepol at 1/10,000 dilution.

Table 3. *Rate of killing of unwashed organisms by copper sulphate*

Time (min.)	Organisms killed in 3×10^{-5}M-$CuSO_4$ In bulk (%)	Organisms killed in 3×10^{-5}M-$CuSO_4$ In drop (%)
1	3 ± 2	—
3	5 ± 2	1 ± 1
5	4 ± 1½	—
6	10 ± 2	2½ ± 1
7	10 ± 2	4 ± 3
10	18 ± 3	4 ± 1½
15	38 ± 2½	—
16	37 ± 2½	9 ± 2
20	56 ± 2	11 ± 3

Table 4. *Effect of adding Teepol to the copper sulphate solution*

	Organisms killed in 15 min. in 3×10^{-5}M-$CuSO_4$ In bulk (%)	Organisms killed in 15 min. in 3×10^{-5}M-$CuSO_4$ In drop (%)
Without Teepol	52 ± 3	23½ ± 2
With Teepol	56 ± 3	55 ± 3

Disinfection with phenol

Phenol was equally toxic to washed or unwashed organisms. The effect of various concentrations of phenol on the organisms in bulk suspensions and in drops was studied in the same way as the copper sulphate. The results are shown in Table 5. It is clear that in the two more dilute concentrations used (0·1 and 0·2%, w/v, phenol) there was markedly more killing in the drops, but with 0·3% (w/v) or stronger phenol this effect was no longer observed. A particularly interesting observation here was that there was actually more

Table 5. *Effect of phenol on organisms in droplets compared with the effect in larger volumes*

Concentration of phenol (%)	Organisms killed in 23 min. In bulk (%)	Organisms killed in 23 min. In drop (%)
0·1	2 ± 2	40 ± 2
0·2	4 ± 2	25 ± 3
0·3	25 ± 3	28 ± 3
0·6	96 ± 2	95 ± 2

killing in the drops with 0·1% (w/v) phenol than there was with 0·2% (w/v) phenol. The killing after various times in 0·1% (w/v) phenol is shown in Table 6.

Table 6. *Rate of killing of organisms by phenol*

	Organisms killed in 0·1% (w/v) phenol	
Time (min.)	In bulk (%)	In drop (%)
0	0	0
5	0	10 ± 3
10	0	40 ± 5
15	0	40 ± 3

DISCUSSION

Any dissolved substance that lowers the surface tension of the drop will be adsorbed so that its concentration in the surface layer is greater than in the rest of the drop (Gibbs's adsorption law; see Bull, 1951). In the case of the experiments with copper sulphate solutions, small amounts of protein were present and these would have concentrated in the surface. As it was found that this protein decreased the toxicity of copper ions, the surface layer of the drop was then less poisonous than the interior. This was confirmed by finding that there was markedly less killing of bacteria in droplets compared with otherwise identical bulk suspensions. The rate of killing was at least twice as great in bulk suspensions as in the drops after about 10 min. If there had been no killing of the bacteria on the surface, then it appears that half the bacteria in the drop must have been on the surface by that time; if there was some killing on the surface, then there must have been rather more than this. It is clear that where the action of a disinfectant is decreased by the presence of protein, droplet suspensions of bacteria may survive concentrations of this disinfectant which are lethal to them when in bulk suspensions.

With phenol, on the other hand, which itself lowers surface tension and is thus concentrated on the surface, the opposite result was found. A concentration of 0·1% (w/v) phenol that was almost without bactericidal action when tested on bulk suspensions proved to kill nearly half the bacteria in 10 min. when in a droplet. Presumably the increased concentration of phenol in the surface layer was sufficient to kill the bacteria that reached and were held there. That a stronger concentration (0·2%, w/v) should kill fewer bacteria in the droplets than the 0·1% concentration seems a paradox, but can be explained as follows. If, as will be suggested later, much of the motion of the organisms is due to their own active movements, then it may well be that the 0·2% (w/v) phenol, though only beginning to show a slight bactericidal action in bulk, might nevertheless have markedly decreased the speed of motion of the bacteria. Thus when it acted on the organisms in the interior of the drop it may actually have served to prevent them rushing to their death in the more toxic surface layer. With the more dilute phenol solution, the bacteria within the drop, because they were moving faster, may have reached and been killed on the still poisonous surface in larger numbers.

If the movement of the bacteria were due entirely to Brownian motion, then the time required for half the population in a drop 0·6 mm. in diameter to reach and be held in the surface can be calculated. The diffusion equation for this case of a spherical drop was solved for the appropriate boundary conditions and showed that the number of bacteria on the surface would rise to half the number originally in the drop in 270 min. (The organisms were almost spherical; for the purposes of this calculation they were assumed to be spheres of diameter 1 μ. and of density 1·1 g. cm.$^{-3}$.) Observations on inanimate objects (polystyrene latex spheres of diameter 0·25 μ.) have shown that their motion under similar conditions conforms exactly to that calculated for bodies in Brownian motion. Now both the copper sulphate and the phenol experiments suggest that in fact about half the bacteria must have reached the surface within 10 min. Their mean energy of motion, which if random is proportional to the time taken for a given fraction of the bacteria to reach the surface thus appears to have been some 25 or more times that of Brownian motion. These bacteria are smooth almost spherical organisms and lack all traces of flagella; nevertheless, it seems that they can move themselves in a given time about 5 (i.e. $\sqrt{25}$) times as far as a similar inanimate object would be moved by Brownian motion.

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REFERENCES

Bull, H. B. (1951). *Physical Biochemistry*, 2nd ed. p. 198. New York: John Wiley and Sons.

Fry, R. M. & Greaves, R. I. N. (1951). The survival of bacteria during and after drying. *J. Hyg., Camb.*, **49**, 220.

Valentine, R. C. & Bradfield, J. R. G. (1954). The urea method for bacterial viability counts with the electron microscope and its relation to other viability counting methods. *J. gen. Microbiol.* **11**, 349.

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HANCOCK, R. (1957). *J. gen. Microbiol.* **17**, 480–490

Protein Synthesis in a Species of Thermophilic Bacillus

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SUMMARY: Washed suspensions of a newly isolated species of thermophilic bacillus were used to investigate the conditions necessary for protein synthesis, and the effect of temperature on the rate of such synthesis. The maximum rate of incorporation of radioactive glycine into the cell protein occurred in the presence of a complete amino acid mixture and an energy source; a mixture of purines and pyrimidines was markedly stimulatory. These conditions were optimal also for the synthesis of catalase and of the maltozymase enzyme-complex, and were effective in preventing the loss of β-galactosidase and catalase activities which occurred when the cells were suspended in buffer at 55°. The synthesis of these enzymes and the incorporation of glycine showed a temperature optimum approximating to that for growth.

The evidence concerning the growth of thermophilic bacteria suggests that at least two mechanisms may be responsible for the ability of these organisms to grow at temperatures at which mesophilic species are rapidly killed. A number of workers have compared the thermal stability of enzymic activities in thermophilic and mesophilic bacteria; Casman & Rettger (1933) and Edwards & Rettger (1937) found some correlation between higher maximum growth temperature and increased thermal resistance of oxidative enzymes among organisms of the genus *Bacillus*; there were, however, numerous exceptions, including some thermophilic strains in which the enzymes studied were no more thermostable than those of the mesophilic group, and in which the 'minimum temperature for enzyme inactivation' was lower than the maximum temperature for growth. Allen (1953) found that the malic dehydrogenase of thermophilic strains of *B. circulans* was more thermostable than that of mesophilic strains; in thermophilic strains of *B. subtilis* this enzyme was no more thermostable than that in mesophilic strains. Rapid inactivation of the catalase and respiratory activities of some thermophilic strains of *B. subtilis* was also found to occur at the optimum growth temperature. In *B. stearothermophilus* some enzymes possess increased thermal stability not only in intact organisms but also in subcellular fractions (respiratory systems) and in purified preparations (hydrolytic enzymes) (Militzer, Sonderegger, Tuttle & Georgi, 1950; Militzer, Tuttle & Georgi, 1951; Militzer & Tuttle, 1952; Militzer & Burns, 1954). Thermal stabilization of an enzyme by association with other proteins occurs in the case of an alanine racemase of *B. terminalis* spores; this enzyme is stable at 80° in whole spores and in cell-free extracts, but completely loses its thermostability when partially purified (Stewart, 1953). Adye & Koffler (1953) showed that the flagella of certain thermophilic

Bacillus species were more resistant to destruction by heat than those from two mesophilic species.

Where no increased thermal resistance of enzymes can be found Allen (1950, 1953) has suggested that the ability of thermophilic organisms to maintain a greater rate of replacement of cell material at high temperatures may be a second mechanism involved in thermophilic growth. Consequently the growth of a thermophilic culture would represent a dynamic equilibrium between rate of thermal inactivation and rate of synthesis of cell components. The present paper describes investigations on the protein-synthesizing systems of a thermophilic *Bacillus* sp. with particular reference to the effects of temperature.

METHODS

Organism and media

The organism used was an aerobic spore-forming *Bacillus* sp. isolated from soil by screening for a spore-forming species capable of growth at 55° but not at 37°. The organism, referred to as Bacillus 631, resembled most closely those of the *B. sphaericus* group in the classification of Allen (1953) and had the temperature/growth-rate characters shown in Fig. 1. The organism was maintained at 55° and transferred at 2 day intervals on slopes of solid medium (pH 7·5) containing tryptic hydrolysate of casein (≡3 % (w/v) casein), 1 % (w/v) glucose, 0·1 % (v/v) Marmite and 3 % (w/v) agar (Harrington Bros. Ltd.).

Growth and preparation of suspensions

For the preparation of washed suspensions the organism was grown in a liquid medium (pH 7·0) containing Oxoid brand peptone (2 %, w/v) + glucose (1 %, w/v), using as inoculum a suspension of organisms washed from a 15 hr. slope culture with 0·85 % (w/v) NaCl. Peptone medium (150 ml.) in 250 ml. round-bottomed flasks was inoculated to give an initial concentration of *c.* 5 mg. dry wt. organism/100 ml. medium. The flasks were shaken for 4 hr. in a water bath at 55°, after which time the culture was growing exponentially and the organisms had divided three or four times (preliminary work had indicated that maximum rates of incorporation of [^{14}C]glycine and of enzyme formation were obtained with organisms harvested from cultures in the exponential phase of growth). For experiments under anaerobic conditions the flasks were fitted with a stopper and tap and anaerobiosis was established by alternate evacuation and filling with N_2; these flasks were also fitted with a side-arm so that growth could be followed by optical density measurements at 450 mμ. using a 'Unicam' spectrophotometer (model 348 DG). The organisms were harvested by centrifugation at 2000 *g* for 15 min., washed once in 0·85 % (w/v) NaCl and then resuspended in 0·85 % (w/v) NaCl to a concentration equivalent to 10 mg. dry wt. organism/ml. This suspension was diluted tenfold in the final incubation mixtures.

The amounts of organism in suspension were determined from optical density measurements using a Hilger 'Spekker' absorptiometer with a neutral filter, and a calibration curve prepared for the organism by drying samples of known optical density to constant weight at 105°. Weights of organism are given throughout in terms of dry weights.

Components of the incubation systems

Amino acid mixture (A) contained the following amino acids at a concentration of 2 mg. of the L-isomer/ml.: L-alanine, L-arginine hydrochloride, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-histidine hydrochloride, DL-isoleucine, L-leucine, L-lysine hydrochloride, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophane, L-tyrosine, L-valine. The solution was adjusted to pH 7·0. Amino acid mixture (B) contained all the above amino acids except glycine. Purine-pyrimidine mixture (PP) contained the following compounds in water at 1·0 mg./ml.: adenine, guanine, xanthine, hypoxanthine, uracil, thymine. To bring these compounds into solution it was necessary to warm the mixture immediately before use. A buffered salts solution (Gale, 1947) was included in all incubation mixtures at a dilution of 1/2.

[α-^{14}C]*Glycine* was obtained from the Radiochemical Centre, Amersham, and was used at a specific activity of 0·1 μC/μmole by dilution with unlabelled glycine. A generally [^{14}C]-labelled protein hydrolysate was obtained by hydrolysis (6N-HCl, 16 hr., 105° in sealed tube) of protein from *Chlorella vulgaris* grown in [^{14}C]O_2 (from the Radiochemical Centre, Amersham); radioautographs of chromatograms of this material showed activity in positions corresponding to all amino acids, with highest activity in glutamic and aspartic acids.

Incorporation of [^{14}C]glycine

The incorporation experiments were carried out in boiling tubes containing buffered salts solution (final dilution 1/2), amino acid mixture (B) (100 μg. of each L-isomer/ml.), mixture PP (10 μg. of each component/ml.), glucose (1 %, w/v) and [^{14}C]glycine at the indicated concentration. Incubation in this system will be referred to as incubation under condition 2; in a similar system without amino acid mixture (B) incubation will be referred to as under condition 1. Components, when omitted, were replaced by water. Organisms were added after the tubes had been equilibrated at the required temperature; tubes were stoppered, and, except where incorporation at a number of different temperatures was being compared, were shaken to improve aeration. The protein fraction of the organisms was prepared as follows: a sample from the incubation mixture was pipetted into one-third of the sample volume of 20 % (w/v) trichloroacetic acid (TCA), centrifuged, extracted three times at 90° for 10 min. with 5 % (w/v) TCA, washed once with 5 % (w/v) TCA containing unlabelled glycine (0·1 %, w/v) and once with 5 % (w/v) acetic acid. The pellet was dissolved in 0·2 ml. of N-NH_4OH, plated on a polythene planchette (2 cm.^{2}), together with one drop of cetyltrimethylammonium bromide (1 mg./ml.) and dried over H_2SO_4 *in vacuo* for 16 hr. The [^{14}C] content of the dried preparations was determined by the use of an end-window Geiger-Muller tube and scaler in the conventional manner. All samples were plated at less than 1 mg./cm.^{2} so that self-absorption was negligible; at least 1000 counts were recorded. Samples of [^{14}C]glycine were plated in a similar manner and counted; activities have been expressed in terms of μmole glycine incorporated. To locate radioactivity in the protein fraction, samples of labelled protein were hydrolysed, submitted to two-dimensional paper chromatography, and radioautographs prepared, by the methods described by Roberts *et al.* (1955). To obtain a clear separation of glycine from serine, samples of the hydrolysate were submitted to paper electrophoresis in 0·05 M-carbonate-bicarbonate buffer (pH 9·6) for 2 hr. at 13·5 V./cm. (Davies, R., personal communication); radioautographs were prepared and components identified by reference to marker amino acids run on the same paper. After incorporation of glycine, in the presence and absence of other amino acids, radioactivity was found only in the glycine residues of the protein.

Incubation mixture for formation of enzymes

The incubation mixture was the same as that used for the incorporation of [^{14}C]glycine except that the complete amino acid mixture (A) was used; other components were added or omitted as described.

Assay of enzyme activities

Catalase was assayed by the method of Creaser (1956). Catalase activities were so high that samples of the incubation mixture were taken into manometers for assay; none of the components of the incubation mixture had any effect on the assay procedure.

β-Galactosidase was assayed by the method of Lederberg (1950) with an incubation period of 15 min. during which the rate of release of *o*-nitrophenol was linear. Ultrasonic breakage of organisms resulted in a 20% increase in the enzyme activity of a suspension of organisms; the ratio of activity of whole organisms : activity of broken organisms did not change in material assayed after incubation under the experimental conditions used.

Maltozymase (defined here as the system responsible for the uptake of O_2 in the presence of maltose and a CO_2-absorbent) was estimated in Warburg manometers; cups contained: 2·3 ml. 0·067 M-phosphate buffer (pH 6·5), 0·5 ml. 0·1 M-maltose and 0·2 ml. 40% (w/v) KOH in the centre well; suspensions of organisms in buffer were added from the side-arm. The gas phase was air and gas uptake was followed at 55°. Activities are expressed as Q_{O_2} values and have been corrected for endogenous gas uptake by organisms in absence of maltose. Manometer joints were greased with silicone high-vacuum grease (Dow-Corning).

Protein nitrogen was determined by the Kjeldahl method followed by microdistillation (Markham, 1942) and nesslerization (Johnson, 1941).

RESULTS

Incorporation of [^{14}C]glycine into cell protein

Fig. 2 shows the rate of incorporation of ^{14}C into the protein fraction of organisms incubated at 55° under different conditions. There was little incorporation in the absence of an energy source; the presence of 17 other amino acids (condition 2) converted the 'plateau' incorporation obtained in their absence (condition 1) into a linear incorporation and under condition 2 a parallel increase in the protein nitrogen of the organisms occurred (Table 1). Under condition 2, different concentrations of glycine gave the results plotted by the method of Lineweaver & Burk (1934) in Fig. 3. In subsequent

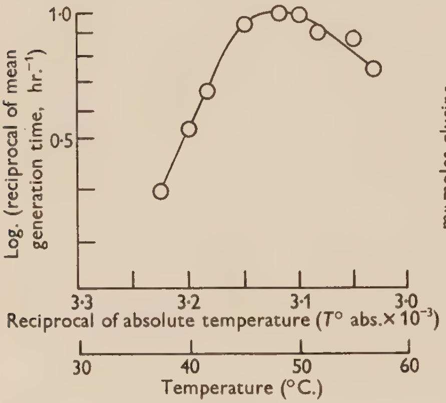

Fig. 1

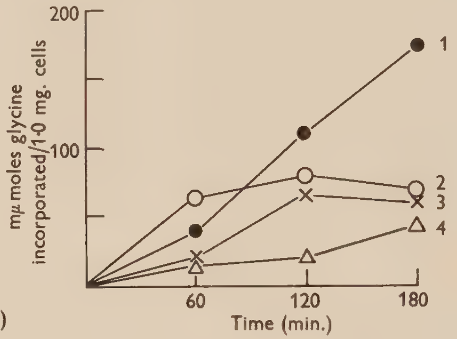

Fig. 2

Fig. 1. Growth rate of Bacillus 631 as a function of temperature. Growth in a medium containing peptone (2 %, w/v) and glucose (1 %, w/v) determined by absorption at 450 mμ in a 'Unicam' model 348DG spectrophotometer.

Fig. 2. Incorporation of [^{14}C]glycine into the protein fraction of Bacillus 631. Incubation medium contained buffered salts, 17 amino acids (100 μg./ml.), purine-pyrimidine mixture (10 μg./ml.), glucose (1 %, w/v) and [^{14}C]glycine at 2 μmole/ml. (specific activity 0·1 μC/μmole). Curve 1, complete system; curve 2, amino acids omitted; curve 3, purine-pyrimidine mixture omitted; curve 4, glucose omitted. Incubation temperature 55°.

Table 1. *Relation between incorporation of [^{14}C]glycine and increase in protein in washed suspensions of Bacillus* 631.

Incubation medium contained buffered salts, 17 amino acids (100 μg./ml.), purine-pyrimidine mixture (10 μg./ml.), glucose (1 %, w/v) and [^{14}C]glycine at 2 μmole/ml. (specific activity 0·1 μC/mole). Radioactivity and total nitrogen were determined on the protein fraction prepared from 2·0 mg. cells.

Incubation time (min.)	Radioactivity (counts/min.)	Increase in protein N (μg.)
30	910	18
60	1640	36
90	2000	54
120	2660	74
180	3810	112

experiments a glycine concentration of 2 μmole/ml. was used to saturate the incorporation system. The magnitude of the response to an increase in the concentration of mixture PP under condition 2 was variable; in a number of experiments the stimulation of incorporation produced by 10 μg. mixture PP/ml. varied from 100 to 300%, and the response was proportional to the concentration of mixture PP (Fig. 4).

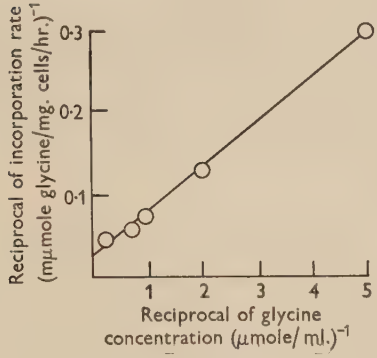

Fig. 3

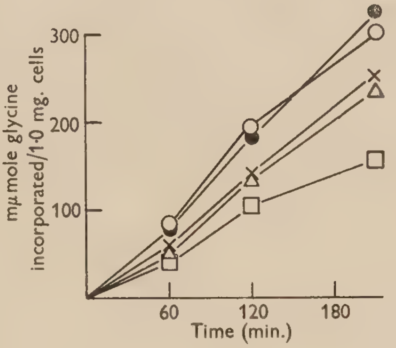

Fig. 4

Fig. 3. Incorporation rate as a function of glycine concentration. Conditions as for Fig. 2, curve 1, but with concentration of glycine as indicated; incorporation determined after 60 min. at 55°.

Fig. 4. Stimulation of rate of incorporation of [^{14}C]glycine into protein fraction by purine-pyrimidine mixture. Conditions as for Fig. 2, curve 1, but with concentration of purine-pyrimidine mixture (μg./ml.) as indicated. (□, 0; △, 2; ×, 5; ●, 10; ○, 20).

The nature of incorporation in absence of other amino acids

The progress curves of incorporation under conditions 1 and 2 were similar to those obtained for the incorporation of glutamic acid into the protein of *Staphylococcus aureus* (Gale & Folkes, 1953*a*), where it was subsequently shown that in disrupted cell preparations (but not in intact organisms) incorporation under condition 1 was reversible in the presence of unlabelled glutamic acid and an energy source (Gale & Folkes, 1955). With intact Bacillus 631 attempts to displace [^{14}C]glycine, after incorporation, by unlabelled glycine were unsuccessful. It is possible that in this case incorporation under condition 1 was due to synthesis of protein from the amino acids of the internal pool; the size of this pool has been investigated by the ninhydrin method (Cocking & Yemm, 1954) and at the time of harvesting is *c.* 0·2 μmole amino acid/mg. dry wt. organism. Two-dimensional chromatograms of the components of this internal pool showed ninhydrin-reacting spots corresponding to all amino acids except histidine; there was no change in the pattern of distribution after hydrolysis. Synthesis of protein from this pool might result in the incorporation of the observed amount of glycine, *c.* 0·08 μmole/mg. dry wt. organism (Fig. 2). The sensitivity of incorporation to inhibition by *p*-fluorophenylalanine (PFP) differed under conditions 1 and 2.

After 2 hr. in the presence of PFP (5 mg./ml.), under condition 1 incorporation was 28·3% of control (724 counts/min.) and under condition 2, 14·5% of control (3619 counts/min.). In *Staphylococcus aureus* incorporation of glutamic acid under condition 1 showed less sensitivity to inhibition by *p*-chlorophenylalanine than did incorporation under condition 2 (Gale & Folkes, 1953*b*).

Effect of temperature on rate of incorporation

Under condition 2 the rate of incorporation of glycine over the range 30°–60° varied as shown in Fig. 5. Between 25° and 55° the temperature coefficient of incorporation was 2·3 (compared with a value from Fig. 1 of 2·8 for growth of the organism). The optimum temperature was higher than that (44°) for the incorporation of glutamic acid in *Staphylococcus aureus* (Gale & Folkes, 1953*a*).

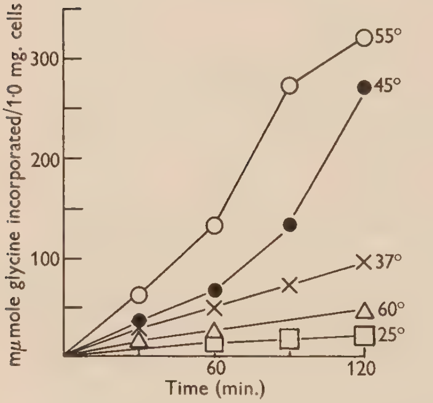

Fig. 5

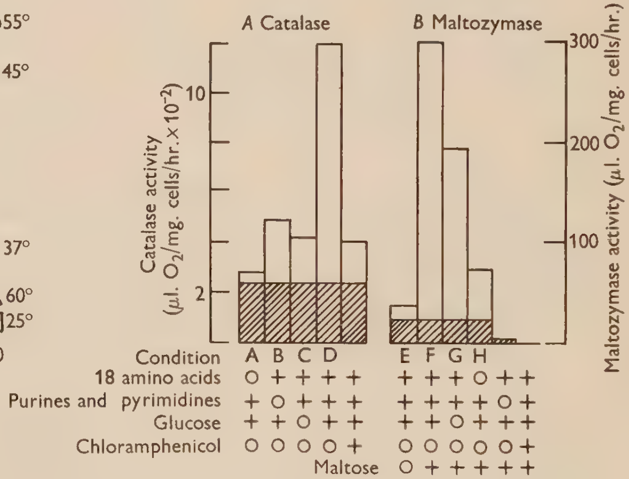

Fig. 6

Fig. 5. Effect of temperature on rate of [^{14}C]glycine incorporation into protein fraction. Conditions as for Fig. 2, curve 1, but with incubation temperature as indicated.

Fig. 6. Development of enzymic activities in washed suspensions of Bacillus 631. Incubation medium contained buffered salts, 18 amino acids (100 μg./ml.), purine-pyrimidine mixture (10 μg./ml.) and glucose (1%, w/v) with omissions as shown and addition of maltose (100 μg./ml.) or chloramphenicol (50 μg./ml.) where indicated. Enzyme activities determined after incubation for 60 min. at 55°; initial activities indicated by shaded areas.

Development of enzyme activities in washed suspensions

In a search for inducible enzyme systems in Bacillus 631 the following enzymes (enzyme activities found to be present in organisms grown under normal conditions are italicized) were investigated: *tetrathionase*, urease, *pyrophosphatase*, arginine dihydrolase, decarboxylases for a number of amino acids, *catalase* and *β-galactosidase*; and oxidation of *lactose*, *galactose*, *arabinose*, *maltose* and *salicin*. The catalase activity of organisms grown under nitrogen was the only case in which large increases in activity were induced in washed suspensions. The replacement of glucose by lactose or maltose in the growth medium resulted in approximately a tenfold increase in the oxidative activity towards the relevant substrate of the organisms from these cultures, but only maltozymase showed consistent increases in activity when washed suspensions were incubated with maltose. Optimum conditions for the development of catalase and maltozymase in washed suspensions are shown in Fig. 6; amino acids, purines and pyrimidines, and an energy source are all required for optimum enzyme formation, omission of any of these reducing formation to one-third or less. The development of both enzymes was inhibited by chloramphenicol at approximately ten times the growth-inhibitory concentration, and it seems probable that the development of both activities was due to synthesis of new enzyme protein.

Effect of temperature on rate of development of enzyme activities

Below 37° the rate of increase of activity was very low in both cases, and the optimum temperature for enzyme formation appeared to be near to that for growth (*c.* 50°) (Figs. 7, 8). It was clearly higher than that found for tetrathionase synthesis (35°–37°) in *Salmonella paratyphi B* (Pollock, 1945) or penicillinase synthesis (*c.* 37°) in *Bacillus cereus* (Knox & Collard, 1952), both of which organisms are considered to be mesophilic.

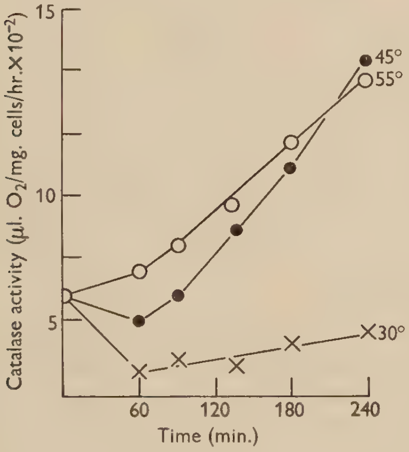

Fig. 7

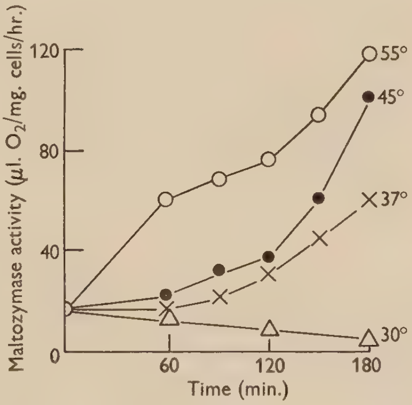

Fig. 8

Fig. 7. Effect of temperature on rate of development of catalase activity. Incubation medium as for Fig. 6, condition D, but with incubation temperature as indicated.

Fig. 8. Effect of temperature on rate of development of maltozymase activity. Incubation medium as for Fig. 6, condition F, but with incubation temperature as indicated.

Incubation of organisms at 55° in buffered salts alone resulted in a decrease in the catalase and β-galactosidase activities whereas these activities showed, respectively, an increase and little change when incubation took place under condition 2; the addition of 100 μg. chloramphenicol/ml. abolished this stabilizing effect of condition 2 (Fig. 9A, B). To determine whether the loss of enzyme activity was due to a general autolytic breakdown of protein, organisms were grown in a medium containing a generally [^{14}C]-labelled protein hydrolysate, and the release of ^{14}C from the protein fraction of organisms incubated in buffered salts alone was followed. No significant release of radioactivity was found. Electron micrographs of organisms after incubation under these conditions show that, whereas in buffer alone most of the organisms lysed and lost electron-dense material, under condition 2 they remained morphologically normal.

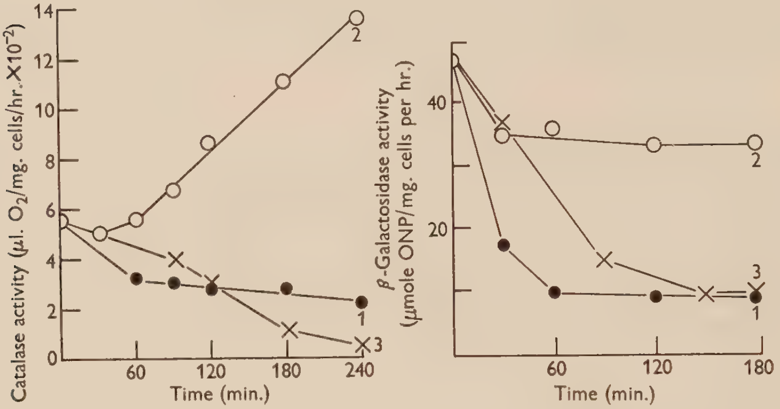

Fig. 9A Fig. 9B

Fig. 9. Effect of nature of the incubation medium on loss of catalase and β-galactosidase activities. Incubation medium contained: curve 1, buffered salts; curve 2, as curve 1 with addition of 18 amino acids (100 µg./ml.), purine-pyrimidine mixture (10 µg./ml.) and glucose (1 %, w/v); curve 3, as curve 2 with the addition of chloramphenicol (100 µg./ml.). Incubation temperature 55°. Fig. 9A, catalase activities. Fig. 9B, β-galactosidase activities.

DISCUSSION

The system responsible for the incorporation of glycine into the protein of Bacillus 631 shows the same response to the presence of a mixture of 17 other amino acids (condition 2) as does the system which incorporates glutamic acid into the protein of *Staphylococcus aureus* (Gale & Folkes, 1953*a*), and in the presence of these amino acids the linear incorporation is accompanied by protein synthesis. It is possible that incorporation when glycine is the only amino acid present (condition 1) ceases when the amino acids of the internal pool are exhausted and net protein synthesis can no longer occur; the situation is complicated by the fact that in the absence of added amino acids most of the organisms lyse, so that the cessation of incorporation may only reflect loss of activity of the incorporation mechanism when the organisms lyse. Because of the failure to find any reversal of incorporation under condition 1, it is not possible to conclude that for Bacillus 631 the mechanisms of incorporation under the two conditions are different.

The requirement for amino acids for the development of enzyme activities, and the effect thereon of chloramphenicol, make it probable that synthesis of new enzyme is occurring under the conditions used. Since the synthesis of certain adaptive enzymes in *Staphylococcus aureus* requires concomitant synthesis of ribonucleic acid (Creaser, 1955, 1956), and is stimulated by purines and pyrimidines, it seems possible that a similar situation obtains in the case of Bacillus 631 and that this is the basis for the marked stimulation of enzyme formation and of glycine incorporation by added purines and pyrimidines.

The substances necessary for the maintenance of β-galactosidase activity are the same as those necessary for the synthesis of other enzymes (compare Fig. 6), and since the presence of chloramphenicol under condition 2 results in a loss of enzyme similar to that which occurs in buffer alone it seems a reasonable deduction that the maintenance of β-galactosidase and the production of catalase are due to synthesis of new enzyme protein which compensates for inactivation. The latter is probably a thermal and not an autolytic process since no loss of radioactivity from the protein fraction was shown.

The experimental results indicate that the protein-synthesizing systems of this thermophilic organism are less thermolabile than those comparable systems which have been studied in mesophilic organisms; nevertheless, it appears that at high temperatures the organism maintains certain enzyme activities by a synthesis which balances thermal inactivation. In this respect the experimental evidence supports the theory put forward by Allen (1953).

The author is indebted to the Medical Research Council for a Scholarship for Training in Research Methods, and to Dr E. F. Gale, F.R.S., and Dr R. Davies for much helpful advice.

REFERENCES

Adye, J. & Koffler, H. (1953). Bacterial flagella as test system for studying heat resistance. *Bact. Proc.* p. 91.

Allen, M. B. (1950). The dynamic nature of thermophily. *J. gen. Physiol.* **33**, 205.

Allen, M. B. (1953). The thermophilic aerobic sporeforming bacteria. *Bact. Rev.* **17**, 125.

Casman, E. P. & Rettger, L. F. (1933). Limitation of bacterial growth at higher temperatures. *J. Bact.* **26**, 77.

Cocking, E. C. & Yemm, E. W. (1954). Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, xii.

Creaser, E. H. (1955). The induced (adaptive) biosynthesis of β-galactosidase in *Staphylococcus aureus*. *J. gen. Microbiol.* **12**, 288.

Creaser, E. H. (1956). The assimilation of amino acids by bacteria. 22. The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus*. *Biochem. J.* **64**, 539.

Edwards, O. F. & Rettger, L. F. (1937). The relation of certain respiratory enzymes to the maximum growth temperature of bacteria. *J. Bact.* **34**, 489.

Gale, E. F. (1947). The assimilation of amino acids by bacteria. 1. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.

Gale, E. F. & Folkes, J. P. (1953*a*). The assimilation of amino acids by bacteria. 18. The incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus*. *Biochem. J.* **55**, 721.

GALE, E. F. & FOLKES, J. P. (1953*b*). The assimilation of amino acids by bacteria. 19. The inhibition of phenylalanine incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine. *Biochem. J.* **55**, 730.

GALE, E. F. & FOLKES, J. P. (1955). The assimilation of amino acids by bacteria. 20. The incorporation of labelled amino acids by disrupted staphylococcal cells. *Biochem. J.* **59**, 661.

JOHNSON, M. J. (1941). Isolation and properties of a pure yeast polypeptidase. *J. biol. Chem.* **137**, 575.

KNOX, R. & COLLARD, P. (1952). The effect of temperature on the sensitivity of *Bacillus cereus* to penicillin. *J. gen. Microbiol.* **6**, 369.

LEDERBERG, J. (1950). The β-D-galactosidase of *Escherichia coli* strain K12. *J. Bact.* **60**, 381.

LINEWEAVER, H. & BURK, D. (1934). The determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658.

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

MILITZER, W. & BURNS, L. (1954). Thermal enzymes. 6. Heat stability of pyruvic oxidase. *Arch. Biochem. Biophys.* **52**, 66.

MILITZER, W., SONDEREGGER, T. B., TUTTLE, L. C. & GEORGI, C. E. (1950). Thermal enzymes. 2. Cytochromes. *Arch. Biochem.* **26**, 299.

MILITZER, W. & TUTTLE, L. C. (1952). Thermal enzymes. 4. Partial separation of an adenosinetriphosphatase from an apyrase fraction. *Arch. Biochem. Biophys.* **39**, 379.

MILITZER, W., TUTTLE, L. C. & GEORGI, C. E. (1951). Thermal enzymes. 3. Apyrase from a thermophilic bacterium. *Arch. Biochem. Biophys.* **31**, 416.

POLLOCK, M. R. (1945). The influence of temperature on the adaptation of 'tetrathionase' in washed suspensions of *Bact. paratyphosum B. Brit. J. exp. Path.* **26**, 410.

ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. B. & BRITTEN, R. J. (1955). Studies of biosynthesis in *Escherichia coli*. *Publ. Carneg. Instn*, no. 607.

STEWART, B. T. (1953). Heat-resistant enzyme from bacterial spores. *Fed. Proc.* **12**, 275.

(*Received* 16 *April* 1957)

KIRKHAM, D. S. (1957). *J. gen. Microbiol.* 17, 491–504

The Significance of Polyphenolic Metabolites of Apple and Pear in the Host Relations of *Venturia inaequalis* and *Venturia pirina*

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SUMMARY: Series of apple and pear shoots were inoculated with the scab pathogens *Venturia inaequalis* and *V. pirina* and injected with polyphenolic metabolites extracted from apple and pear leaves, and with urea. The translocation and persistence of arbutin in Cox leaves was demonstrated chromatographically. Injection of metabolites of the highly susceptible varieties Cox and Williams back into the same varieties, markedly inhibited the pathogens, which suggests that these potential resistance factors do not occur only in varieties which are scab resistant. Inhibition of infection was most marked on leaves already developing a mature resistance and the injection treatments were most effective when applied near the time of inoculation. Injection of urea increased susceptibility to *V. inaequalis*, and further results indicated the importance of optimal balance mechanisms between polyphenolic and nitrogenous metabolites. Chlorogenic acid and *iso*chlorogenic acid strongly inhibited both pathogens and there was some indication of synergism between them as resistance factors.

Results obtained in artificial culture (Kirkham, 1957*b*) have shown that polyphenolic host metabolites in relation to other nutritional factors are of potential significance in the scab diseases of apple and pear, caused by *Venturia inaequalis* (Cke.) Wint. and *V. pirina* Aderh., respectively. To obtain more direct evidence of the importance of these compounds, injection trials were carried out on series of inoculated apple and pear shoots under glass. Roach (1934) obtained results in which the incidence of mildew (*Podosphaera leucotricha*) on M. III rootstocks was decreased by injection of a solution of sodium thiosulphate, thus demonstrating the potential value of a plant injection technique in studies of disease reaction. In the present study the Roach method of petiole injection (1938) was used in an attempt to induce variations, both qualitative and quantitative, in the polyphenolic and nitrogenous metabolites within series of similar apple and pear shoots so that effects of these variations on the incidence of the diseases could be observed. Some results of this study have been reported (Kirkham, 1954).

METHODS

Methods similar to those described in a previous paper (Kirkham, 1957*a*) for the determination of host ranges and pathogenicity of distinct clones of *Venturia inaequalis* and *V. pirina* were used. The trees to be used for the season's work were moved into the greenhouse serially, as three groups, between the beginning of March and the end of May, to provide material for three successive inoculation and injection trials during the spring and early summer. On completion of each trial the four shoots on each tree were cut back to the first well-developed bud and, conditions permitting, these buds produced shoots which were ready for use later in the season. Thus a succession of shoots was maintained from May to October or November. To increase further the length of the period in which trials could be carried out, shoots were raised under artificial light in a heated greenhouse. After their first season of growth, trees were chilled at 5° during December and then transferred, after pruning, to a small greenhouse heated to a minimal temperature of 13°. Groups of twenty-four trees were illuminated with one 500 W. tungsten filament lamp and two 400 W. mercury vapour lamps to give a minimal day length of 16 hr. In these conditions trees of Cox's Orange Pippin and Williams' Bon Chrétien produced visibly normal shoots between 7 and 12 in. long by the middle of February.

Monoconidial isolates of each pathogen were used as sources of inoculum. Cultures of *Venturia inaequalis*, clones E1, A7 and A20 isolated from the varieties Edward VII, Worcester Pearmain and Laxton's Superb respectively, and *V. pirina*, clones W1 and W3 isolated from Williams' Bon Chrétien, were grown on filter paper-cylinders standing in 10% (w/v) malt-extract solution (Kirkham, 1956). The cultures of *V. inaequalis* were used directly for the inoculation of the main trials. Inoculum of *V. pirina* was finally prepared from leaves of Williams after two serial transfers of the pathogen on this variety, because of poor sporulation in culture and loss of pathogenicity during storage.

The immature leaves at the tip of each shoot were inoculated. In the first series of trials (1953) the designation L_0 was applied to the first fully unrolled leaf below each shoot tip, but in the second series (1954) when the disease reactions of leaves approaching maturity were under investigation, this designation was applied to the second fully unrolled leaf. Leaves below this point were numbered -1, -2, etc., and above, $+1$, $+2$, etc. Leaf L_0 in the second series is therefore equivalent to leaf -1 in the first series. Three shoots on each tree were inoculated and the fourth remained as an uninoculated control. Each shoot was enclosed in a polythene-sleeve moist chamber and inoculated with a conidial suspension by using a glass throat atomizer. This method of inoculation was adopted to secure a more even distribution of conidia on the leaves than had been achieved in previous studies (Kirkham, 1957*a*). A further improvement was obtained in 1954, when leaves $+1$ to -1 on apple shoots, and leaves $+2$ to -2 on pear shoots, were each sprayed separately. The inoculum concentrations were visually adjusted to a turbidity (Spekker drum reading) of *c.* 0·1 and approximately 1 ml. was used for each shoot.

Shoots were injected either before or after inoculation with 1% (w/v) aqueous solutions of extracts of the polyphenolic metabolites of Cox's Orange Pippin and Williams' Bon Chrétien (the Cox 3 and Williams 3 extracts described by Kirkham, 1957*b*), pure components of these extracts, or urea. Preliminary attempts were made to assess effects of these components and mixtures on the incidence of the diseases after injection of inoculated shoots through single petioles (leaf -2) but no significant differences were observed between disease reactions on permeated and non-permeated leaf zones, owing to the uneven distribution of inoculum. It was therefore necessary to induce complete permeation of each shoot tip, and to compare disease incidence on a series of shoots. With an aqueous solution of acid fuchsin (1%, w/v), it was found that complete permeation resulted from injection through three petioles. Thus, in the trials described below, leaves -2, -3 and -4 were used on apple shoots and -3, -4 and -5 on pear shoots. The laminae were removed from the leaves and the petioles dipped into the injectant contained in polythene tubes wired on to the shoots. Nine shoots on three trees were used in each injection treatment. The solutions were Seitz-filtered before use to avoid blockage of the vessels by solid particles during injection. Between 3 and 5 ml. of injectant were usually taken in by each shoot, depending on the nature and concentration of the solution and the external conditions during injection. The following conditions were necessary for maximal intake:

(*a*) A greenhouse temperature of 18–24°. Below 18° intake was very slow and above 24° too rapid, the latter resulting in wilting and damage to the shoots. (*b*) Moderate air turbulence to increase transpiration; this was achieved by the use of an electric fan. (*c*) A light intensity sufficiently high to maintain active photosynthesis. During 1953 much difficulty was experienced in producing these conditions at the required times and therefore, during the following year, two 500 W. tungsten and four 400 W. mercury vapour lamps were used to provide illumination during injection.

Disease incidence, on a series of shoots to which various injection treatments had been applied, was observed by visual estimation of lesion development at frequent intervals after the first appearance of lesions. Both surfaces of each inoculated leaf were recorded separately by estimation of % surface area covered by sporulating lesions. Leaves -1, L_0 and $+1$ were recorded on apple shoots and leaves -2 to $+2$ on pear shoots as more leaves usually became diseased on the latter. In each treatment nine replicate leaves (eighteen surfaces) were recorded in each leaf category, and the average for the category was described as the *mean percentage lesion cover*. Observations were also made on fleck reactions and on leaf damage due to various causes. The results are illustrated graphically, relating mean percentage lesion cover to the time (in days) after inoculation, for separate leaf numbers. The relationship between the probit values of the mean percentages and the logarithms of the times appeared to be approximately linear and so probit and logarithm scales were used for convenience. With one exception (trial 3/54), large differences resulted from the various treatments, and a high degree of correlation was observed between the results of similar trials carried out on separate occasions. In these circumstances the results, excepting those of trial 3/54, were not analysed statistically.

The chromatograms were run as previously described (Kirkham, 1957*b*).

RESULTS

Inhibition of Venturia inaequalis *and* V. pirina *on leaves injected with polyphenolic host metabolites*

Cox trees on M. IV rootstocks were inoculated with *Venturia inaequalis*, clone E1. Twenty-four hr. later nine shoots were injected with the Cox extract (Cox injection 1), nine shoots with water, and eighteen were left uninjected. Twelve days after inoculation, when lesion traces were visible, nine of the uninjected shoots were treated with the same Cox injectant (Cox injection 2). The volume taken up by each shoot was approximately 2·5 ml. Symptoms of iron deficiency were visible in the material and the trees were arranged, as far as possible, to include all stages of severity in each treatment. No damage resulted from the injections, but some leaves were lost by sun-scald during inadequate shading of the greenhouse. Fig. 1 illustrates the incidence of disease recorded on −1 leaves in this trial (no. 4/53). The first Cox injection markedly reduced lesion development but the second, 12 days after inoculation, was less effective. Leaves in the L_0 category showed similar disease reactions but there was less inhibition and less effect of time of injection. No inhibition was observed in the water treatment.

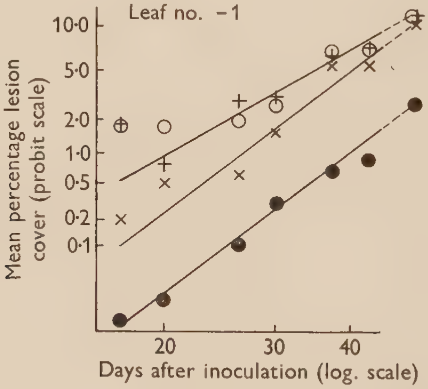


Fig. 1. Inhibition of *Venturia inaequalis*, clone E1 on Cox, by injection of polyphenolic metabolites of Cox (Trial 4/53). Cox 1: injected 24 hr. after inoculation; Cox 2: injected 12 days after inoculation. Injections: +, no injection; ○, water; ●, Cox 1; ×, Cox 2.

Trial 10/53 showed similar reactions of *Venturia pirina* (clone W1) on Williams' pear. Twenty-four hr. before inoculation nine shoots were injected with the Williams' extract and nine shoots with water. The intake of each injectant was approximately 3 ml./shoot and no leaf damage was observed. Figs. 2–4 show the reactions observed on −2, −1 and L_0 leaves respectively. Considerable inhibition of the pathogen occurred on −2 leaves but on L_0 leaves where the mean % lesion cover on the controls was considerably higher, only very slight inhibition resulted from the injection of host metabolites. Disease incidence on −1 leaves was intermediate between reactions on −2 and L_0 leaves. These results confirmed indications from small-scale trials on trees raised under artificial light, where inhibition of *Venturia inaequalis*, clones E1 and A26 on Cox and *V. pirina*, clones W1 and P16 on Williams had been observed.

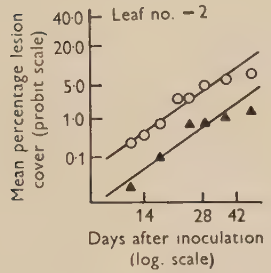


Fig. 2

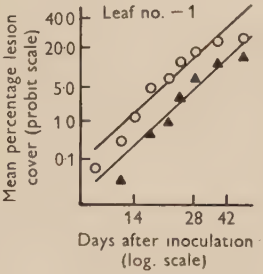


Fig. 3

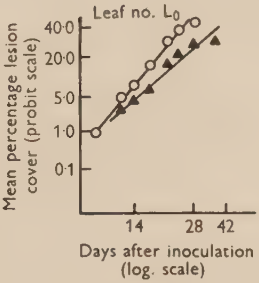


Fig. 4

Figs. 2–4. Inhibition of *Venturia pirina*, clone W1 on Williams' pear, by injection of polyphenolic metabolites of Williams. (Trial 10/53.) Leaves −2, −1 and L_0 in decreasing order of age at time of inoculation. Injections: ○, water; ▲, Williams.

The question arises as to whether or not these reactions resulted from the actual presence of injected compounds in the leaves. The situation is unusual in that compounds already present were injected, and it therefore seems reasonable to suppose that translocation might occur, and that concentration increases might be tolerated to some extent. Very rapid host reactions to the injection treatments were sometimes observed, including interveinal desiccation of mature leaves below the injection points. A temporary return to the juvenile pink colour in leaves of Durondeau pear after injection of polyphenolic metabolites was also noted. These observations, however, do not necessarily indicate that translocation occurred unless they can be supported by direct chemical evidence. To obtain this evidence apple seedlings were injected with the pear glycoside arbutin. Twenty-four hr. later, leaves above the injection points were removed and their polyphenolic fraction compared chromatographically with that of uninjected leaves; it was found that the foreign glycoside was present in the injected leaves. It will therefore be assumed, pending further studies of translocation and persistence, that the observed effects on disease incidence resulted from at least the temporary presence of injected compounds in the leaves. Persistence for long periods seems to be unlikely, as the metabolic processes of a leaf would tend to remove foreign compounds, or restore to normal any altered balances between metabolites.

Results obtained in artificial culture (Kirkham, 1957*b*) showed that polyphenolic host metabolites may play an important part in the nutrition of the scab pathogens and the trials described above indicate the possible significance of these compounds in host resistance. The incidence of the diseases on Cox apple and Williams' pear was considerably decreased by injection of polyphenolic metabolites which are already present in the highly susceptible leaves.

It seems that these decreases can be most easily induced on leaves which are already developing a mature resistance and, though this effect may be partially due to concentration of injected compounds above the injection points, later results (see below) showed that large differences in disease reaction could also be obtained on leaves above L_0.

Further reactions of Venturia inaequalis *to injections of polyphenolic metabolites of Cox's Orange Pippin*

In trial 6/53, injection towards the end of the incubation period of the disease did not affect lesion development on any leaf. Trees of Cox and Worcester on M. II rootstocks were inoculated with *Venturia inaequalis*, clone E1 and, 12 days later, injected with Cox 3 extract. Fig. 5 shows that there was no inhibition on −1 leaves of Cox, and similar results were obtained with all other leaves in this trial, irrespective of age and degree of lesion cover on the controls. These results confirm those of trial 4/53, where injection 12 days after inoculation was less effective than injection 24 hr. after inoculation. It seems that the pathogen is most susceptible to inhibition during the very early stage of the incubation period of the disease, after penetration of the leaf cuticle and when the development of the subcuticular mycelium is beginning. Similarly, final results in culture were often partially determined by reactions which occurred during the lag phases.

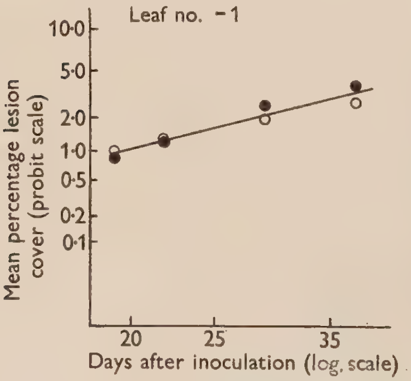


Fig. 5

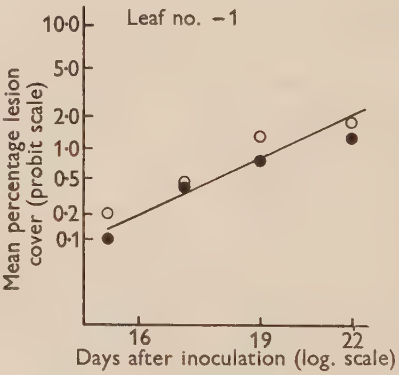


Fig. 6

Figs. 5–6. Lack of inhibition of *Venturia inaequalis*, clone E1 on Cox and clone A20 on Laxton's Superb, by injection of polyphenolic metabolites of Cox 12 days after and 2 days before inoculation respectively. (Trials 6/53 and 9/53.) Injections: ○, water; ●, Cox.

Trial 9/53 also yielded results in which disease incidence was not influenced by Cox 3 extract injection. Trees of Laxton's Superb were inoculated with *Venturia inaequalis* clone A20, originally isolated from this variety, 24 hr. after injection. A high injection intake of approximately 4·5 ml./shoot was observed, but there was no inhibition even on −1 leaves (Fig. 6). In the same trial (9/53) Worcester trees were inoculated with clone E1, 24 hr. after injection. The intake of each injectant was again approximately 4·5 ml./shoot. In contrast to the reaction of clone A 20 on Laxton's Superb, striking inhibition of clone E 1 was observed (Figs. 7, 8). The mean % lesion cover of the L_0 control leaves was higher than that of the −1 control leaves and as expected, less inhibition was observed on L_0 leaves.

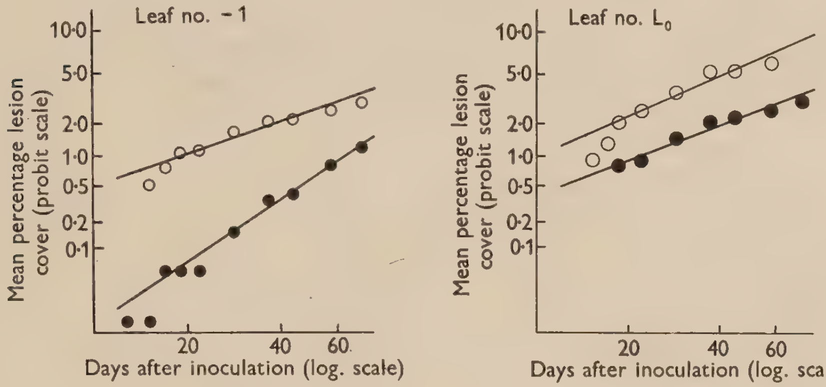


Fig. 7

Fig. 8

Figs. 7–8. Inhibition of *Venturia inaequalis*, clone E 1 on Worcester Pearmain, by injection of Cox metabolites 24 hr. before inoculation (trial 9/53). Injections: ○, water; ●, Cox.

It is clear that injection of polyphenols near the time of inoculation does not necessarily cause inhibition of the pathogen. The occurrence and degree of inhibition will presumably be determined by the particular association of clone and host variety, and does not depend solely on the time of injection. In the case of clone A 20 on Laxton's Superb any quantitative changes induced by injection were insufficient to influence the development of the pathogen. In the same trial, however, the growth of clone E 1 on Worcester was strongly influenced by the injection of Cox metabolites, which again suggests that metabolites contributing to the mechanism of disease resistance can be obtained from highly susceptible as well as less susceptible varieties.

In a previous paper, relationships were demonstrated between the host ranges of distinct clones of *Venturia inaequalis* and their susceptibility to inhibition by the Cox extract, in artificial culture (Kirkham, 1957*b*). Clone E 1 had the widest host range and was least susceptible, while the converse was found for clone A 7, and clone A 20 held an intermediate position. In trial 8a/53 reactions of clones A 20 and A 7 on Cox leaves to injection of the shoots with Cox 3 extract, were observed. Shoots of Cox on M. IV were inoculated, and injected twice with Cox 3 extract during the incubation period of disease, to introduce the maximum volume of injectant into each shoot tip. Twenty-four hr. after inoculation the intake was approximately 3·3 ml./shoot, and 6 days after inoculation, 5 ml./shoot. Owing to lack of sun there was an enforced gap of 5 days between the two injections. Symptoms of iron deficiency, which appeared to be increased by the Cox 3 extract injection, were noted in the material and a complete range of severity was included in each treatment.

Figs. 9 and 10 show reactions of clones A20 and A7 respectively on −1 leaves. Lesion development of both clones was inhibited and, in contrast to the *in vitro* results clone A20 was more susceptible than was clone A7. The high injection intake in this trial did not result in increased inhibition of the pathogen, possibly owing to the fact that the second injection treatment was applied too long after the initiation of the disease. If, on the other hand, the second injection was effective, clones A20 and A7 were less susceptible to inhibition in trial 8a/53 than was clone E1 in trial 4/53, on Cox. There appeared to be no relationship between the host range of a clone and its reaction to injection of a highly susceptible variety with Cox 3 extract.

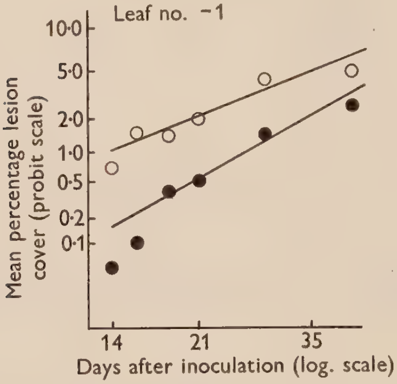


Fig. 9

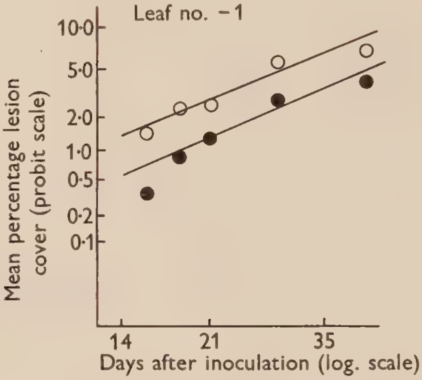


Fig. 10

Figs. 9–10. Inhibition of *Venturia inaequalis*, clones A20 and A7 on Cox, by two injections of Cox metabolites during the incubation period of the disease (trial 8a/53). Injections: ○, water; ●, Cox.

Effects of urea and polyphenolic metabolites on the incidence of Venturia inaequalis *on Cox*

Trees of Cox on M. II rootstocks were inoculated with *Venturia inaequalis* clone E1, and 5 days later shoots were injected with water, Cox 3 extract, a 0·5% (w/v) solution of urea, or a solution containing 1% (w/v) Cox 3 extract + 0·5% (w/v) urea. The intake of each injectant was approximately 3·3 ml./shoot. Injection of urea resulted in marginal scorch which developed slowly on young leaves above the injection points, and during intake of Cox 3 extract rapid interveinal desiccation of leaves below the −2 stage was observed. Pronounced interveinal scorch was visible on these Cox 3 extract treated leaves 12 hr. after injection. The mixture induced slight symptoms of damage by urea and Cox 3 extract but was considerably less phytotoxic. Figs. 11 and 12 show disease incidence on −1 and L_0 leaves respectively (trial 8b/53). Cox 3 extract markedly reduced lesion development on −1 leaves and urea had the opposite effect. In the latter treatment many diffuse lesions were present on the undersurfaces of the leaves. The mixture was intermediate in activity between urea and the water control but, 28 days after inoculation the mean % lesion areas in the mixture and water injection treatments were the same. Disease incidence was not higher on L_0 leaves in this trial, but the Cox 3 extract treatment was, as usual, less effective on these leaves. The urea and mixture injections equally stimulated lesion development on the younger leaves.

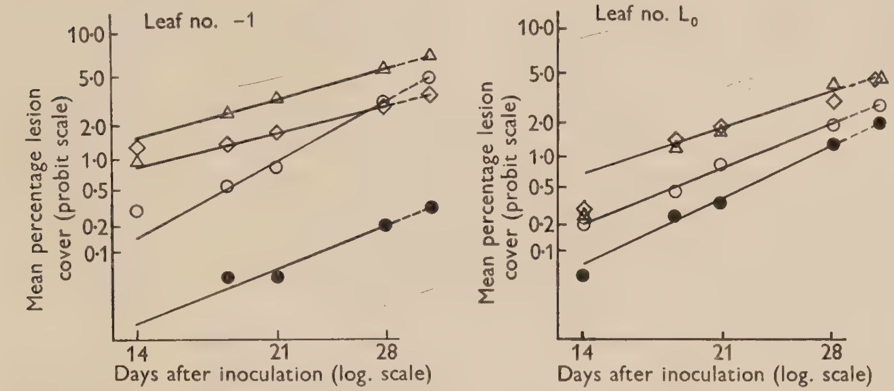


Fig. 11 Fig. 12

Figs. 11–12. Inhibition and stimulation of *Venturia inaequalis*, clone E1 on Cox, by injection of Cox metabolites, urea and a mixture of these two injectants, 5 days after inoculation (trial 8b/53). Injections: ○, water; ◇, Cox and urea; ●, Cox; △, urea.

Previous results obtained in artificial culture (Kirkham, 1957*b*) showed that the activity of the polyphenols was influenced by the composition of the basal medium; inhibition was least marked in the presence of an optimal nutritional substrate. Injection of urea would rapidly result in an enrichment of the nitrogenous constituents of the *in vivo* substrate and might therefore, as suggested by this trial, decrease the inhibition of the pathogen by the naturally occurring polyphenols. In trial 8b/53 inhibition by Cox 3 extract was certainly decreased by the injection of urea. There was some indication that the separate effects of the extract and urea might exactly counteract each other, depending on the proportions in the mixture and the age of the leaf. As indicated by the L_0 result, less urea would be required to achieve this in younger leaves which naturally contain more nitrogen.

Figs. 13–15 illustrate the results of trial 3/54 in which the same injectants as in trial 8b/53 and a 1% (w/v) solution of arbutin were used. Trees of Cox on M. IV rootstocks were inoculated with *Venturia inaequalis* clone E1 2 days before injection. The intake of arbutin was approximately 3 ml./shoot, and of the other injectants approximately 4 ml./shoot. Urea again caused some marginal scorch on the young leaves and the mixture of Cox 3 extract and urea was less damaging but no interveinal desiccation occurred during injection of Cox 3 extract alone. The rate of intake of arbutin, the main pear glycoside, was slow compared with the other injectants and there was no indication of phytotoxicity. In contrast to all previous results, injection of Cox 3 extract increased the incidence of disease on leaves −1 to +1. (Leaf L_0 in the 1954 series of trials is equivalent to leaf −1 in the 1953 series.) It was also noted that the mean % lesion areas on the water-injected shoots in this trial were considerably higher than those resulting from previous combinations of clone E1 and Cox. As relatively small differences between the results of treatments were recorded in trial 3/54, the separate lines on each graph indicate results, or groups of results which differed significantly at the 1% level. There was no significant interaction (5% level) between days after inoculation and treatments. Cox 3 extract stimulated lesion development equally on all leaves, irrespective of their ages and the disease incidence on the controls. Urea was most stimulatory to the pathogen in leaves approaching maturity and its activity was similar to that of Cox 3 extract at the −1 stage, while the activity of the mixture was similar to that of urea but somewhat greater. There was some indication that the mixture was more active than either Cox 3 extract or urea alone in −1 leaves, and it was less active than Cox 3 extract alone in +1 leaves. Arbutin was inactive, inhibitory or stimulatory, depending on leaf age.

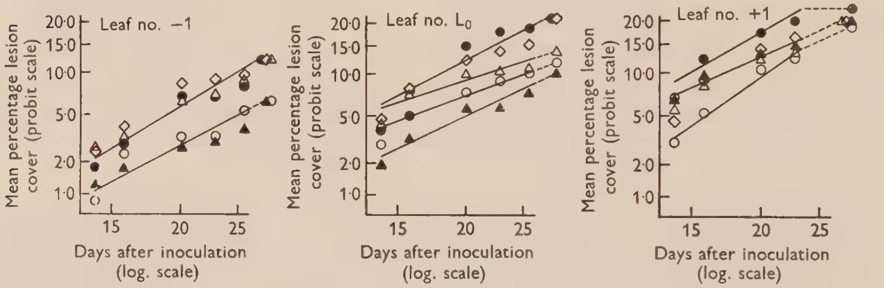


Fig. 13 **Fig. 14** **Fig. 15**

Figs. 13–15. Stimulation of *Venturia inaequalis*, clone E1 on Cox, by injection of Cox metabolites, urea, and a mixture of these two injectants, 2 days after inoculation, and inhibition and stimulation by arbutin (trial 3/54). Injections: ○, water; ●, Cox; △, urea; ◇, Cox and urea; ▲, arbutin.

The results of trial 8b/53 suggested a fairly simple situation in which the reaction of a clone of *Venturia inaequalis* to the ratio between the polyphenolic and nitrogenous metabolites of an apple variety might be an important factor in the determination of pathogenicity. Trial 3/54, however, showed that the polyphenols may stimulate as well as inhibit the pathogen *in vivo*. Similar results have been obtained with *V. pirina in vitro*, but stimulation of *V. inaequalis* has not yet been recorded in the artificial environment. The shoots used in trial 3/54 were growing very rapidly at the time of inoculation, and therefore −1 and L_0 leaves may have been similar to +1 leaves in physiological age and all highly susceptible to the disease, even though L_0 leaves were equivalent to −1 leaves in trial 8b/53. The injection of Cox metabolites resulting in increased polyphenolic content, perhaps up to a critical concentration above which inhibition would occur, seems to have increased the incidence of disease. A quantitative increase in the nitrogenous metabolites, also up to a critical concentration, might increase the stimulatory effect of the naturally occurring polyphenols. This effect would, as observed, be less marked on younger leaves which naturally contain more nitrogen. The mixture of Cox 3 extract and urea was also less active in younger leaves, which suggests that an adverse balance between polyphenolic and nitrogenous metabolites was again established, compared with the balance resulting from injection of Cox 3 extract alone; stimulation was thus reduced. In conditions where Cox 3 extract increased disease incidence on L_0 leaves, arbutin had the opposite effect. Although arbutin is not a natural metabolite of the apple, this fact suggests the potential importance of qualitative differences between the polyphenolic metabolites of varieties and leaves of different ages which are characterized by different degrees of resistance.

Effects of polyphenolic metabolites of Williams' pear, including chlorogenic acid and iso*chlorogenic acid, on* Venturia inaequalis *and* V. pirina *on apple and pear shoots, respectively*

Trial 1/54 was carried out during February on shoots of Cox on M. IV rootstocks, grown under artificial light in the heated greenhouse. Two days after inoculation with *Venturia inaequalis* clone E1, three injection treatments were applied. A 1% (w/v) solution of Williams 3 extract and 0·2% (w/v) solution of chlorogenic acid were used in addition to the water control and approximately 4 ml. were taken in by each shoot. The rate of intake of Williams 3 extract was slow and variable and leaves above the injection points soon began to show symptoms of phytotoxicity; the midribs became arched, the laminae crinkled and the margins desiccated. Three days after injection the marginal desiccation was visible as blackened areas similar to scorch symptoms on pear leaves. Severely damaged leaves became stunted but shoot extension continued with the production of normal leaves. Intake of chlorogenic acid was rapid, and resulted in similar though much less severe damage. Pl. 1 shows leaves from this trial injected with water, chlorogenic acid, and Williams 3 extract, respectively. The phytotoxic action of the Williams extract seemed to be at least partly due to the chlorogenic acid component, even though this depside is naturally present at a low concentration in Cox leaves. This reaction to injection of Williams 3 extract is in striking contrast to the tolerance shown towards Cox 3 extract, and the pear glycoside arbutin (see above). Fig. 16 shows the results of this trial, which again suggest that chlorogenic acid is a highly active metabolite. Owing to decrease of leaf area, disease incidence in the Williams-extract treatment was unavoidably rated somewhat too highly, and is therefore not exactly comparable with the other treatments. Similar results were observed on L_0 leaves.

Some effects of the depsides chlorogenic acid and *iso*chlorogenic acid were observed in trial 2/54. Shoots of Cox on M. IV rootstocks were grown under artificial light and the trees were transferred to an unheated house before inoculation. Two days after inoculation with *Venturia inaequalis* clone E1, four injection treatments were applied. These included, apart from the water control, solutions of the depsides (0·2%, w/v) and another solution containing 0·1% of each compound. Owing to the insolubility of pure *iso*chlorogenic acid in water, it was dissolved in a minimal volume of absolute ethanol before addition of water; all the injectants including the water control therefore contained 1 % (w/v) ethanol. The intake of each injectant, except the depside mixture, was approximately 4 ml./shoot. The depside mixture was injected during cool evening conditions and only *c.* 3 ml. of the solution were taken up by each shoot. Damage following chlorogenic acid injection was again observed, including in this trial some slight interveinal scorch. *iso*Chlorogenic acid was less phytotoxic than chlorogenic acid but induced similar symptoms on −1 leaves. Younger leaves in this treatment slowly developed a slight marginal scorch and a persistent interveinal dark red coloration. Very slight symptoms of chlorogenic acid damage were visible on the mixture-treated shoots, and there was no host reaction to the control injection. Figs. 17–19 show that the depsides inhibited lesion development on leaves −1 to +1 and that their relative activities varied markedly with leaf age. The mixture of chlorogenic acid and *iso*chlorogenic acid was inactive at the −1 stage of leaf

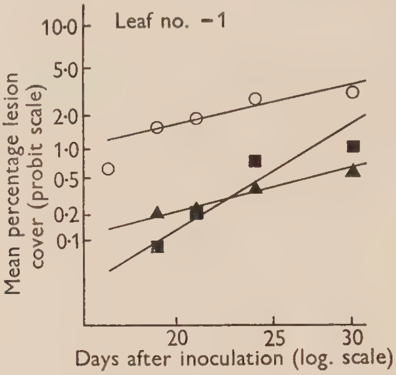


Fig. 16. Inhibition of *Venturia inaequalis*, clone E1 on Cox, by injection of polyphenolic metabolites of Williams' pear and chlorogenic acid alone, 2 days after inoculation (trial 1/54). Injections: ○, water; ▲, Williams; ■, chlorogenic acid.

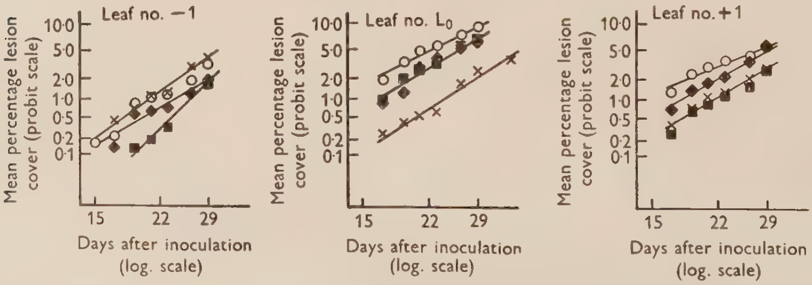


Fig. 17 Fig. 18 Fig. 19

Figs. 17–19. Inhibition of *Venturia inaequalis*, clone E1 on Cox, by injection of chlorogenic acid and *iso*chlorogenic acid as separate and combined injectants, 2 days after inoculation (trial 2/54). Injections: ○, water; ■, chlorogenic acid; ◆, *iso*chlorogenic acid; ×, chlorogenic acid and *iso*chlorogenic acid.

development, strongly inhibitory at the L_0 stage and equal in activity to chlorogenic acid at the +1 stage.

Reactions of *Venturia pirina*, clone W3, on shoots of Williams' pear injected with chlorogenic acid or *iso*chlorogenic acid were observed in trial 4/54 (Fig. 20). Three days after inoculation the same injection treatments were applied as in trial 2/54, but the mixture of chlorogenic acid and *iso*chlorogenic acid was omitted. The intake of each injectant was approximately 3 ml./shoot and there was no leaf damage.

As expected, lesion development on the control shoots varied markedly with leaf age, +1 leaves being most susceptible to the pathogen. Chlorogenic acid was inactive at the +1 and L_0 stages, and slightly inhibited the early development of the pathogen on −1 leaves. *iso*Chlorogenic acid, however, inhibited clone W3 at all leaf stages but was least active in +1 leaves. Thus, in contrast to the reactions of *Venturia inaequalis* on Cox shoots (trial 2/54), *iso*chlorogenic acid was more active against *V. pirina* clone W3 than was chlorogenic acid.

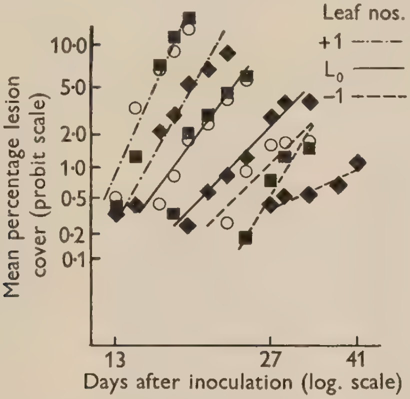


Fig. 20. Inhibition of *Venturia pirina*, clone W3 on Williams, by injection of chlorogenic acid and *iso*chlorogenic acid, 3 days after inoculation (trial 4/54). Injections: ○, water; ■, chlorogenic acid; ◆, *iso*chlorogenic acid.

The results of these three trials indicate that the depsides may be important factors in the host relations of *Venturia* species and that synergism may occur between them as resistance factors. It is thought that trial 4/54 illustrates the results of alterations in the natural chlorogenic acid : *iso*chlorogenic acid ratios in the leaves of Williams' pear and that this ratio may be a significant factor in disease resistance.

DISCUSSION

The results confirm and extend the hypothesis that polyphenolic host metabolites may be important factors in scab resistance. There are further indications of optimal balance requirements, both within and between the polyphenolic and nitrogenous metabolites, for the establishment of a progressive relationship between host and pathogen. The polyphenols may act as limiting factors in nutrition as suggested by the gradations in response, from stimulation to inhibition, which have been observed both in the present studies and in artificial culture. The stimulation of *Venturia inaequalis* resulting from the urea injections supports the conclusions of Johnstone (1931) and of Winkelmann, Holz & Jaenichen (1937) that a high nitrogen concentration increased susceptibility to apple scab. It is well known that mature leaves of all varieties are immune to infection and that this immunity cannot be explained by the thickness of the cuticular barrier alone. Fleck reactions indicating failure of the pathogens after successful infections were more commonly seen on leaves approaching maturity than on young leaves, and the injection treatments were often more effective on the former. This seems to indicate that, in leaves developing a natural mature resistance, it is easier to induce a balance between metabolites which is less favourable to the pathogen. There appears to be a considerable measure of agreement between the results obtained in artificial culture (Kirkham, 1957*b*) and in the natural environments of the pathogens. It seems, therefore, that valuable clues to the mechanisms of scab resistance could be obtained from further studies of the pathogens in pure culture.

The author is indebted to Dr R. V. Harris for advice and interest, to Dr W. A. Roach for placing at his disposal a very wide knowledge of plant injection methods, to Dr A. E. Flood for his continued help, particularly in running the chromatograms, to Mr M. M. MacNeill for raising trees under artificial light, to Miss Jean Richardson for technical assistance and to Dr S. C. Pearce for the statistical analysis.

REFERENCES

JOHNSTONE, K. H. (1931). Observations on the varietal resistance of the apple to scab (*Venturia inaequalis* Aderh.) with special reference to its physiological aspects. Part II. *J. Pomol.* 9, 195.

KIRKHAM, D. S. (1954). Significance of the ratio between the water-soluble aromatic and nitrogen constituents of apple and pear in the host-parasite relationships of *Venturia* species. *Nature, Lond.* 173, 690.

KIRKHAM, D. S. (1956). A culture technique for *Venturia* spp. and a turbidimetric method for the estimation of comparative sporulation. *Nature, Lond.* 178, 550.

KIRKHAM, D. S. (1957*a*). Relationships between cultural characters and pathogenicity in *Venturia inaequalis* and *Venturia pirina*. *J. gen. Microbiol.* 16, 360.

KIRKHAM, D. S. (1957*b*). Studies of the significance of polyphenolic host metabolites in the nutrition of *Venturia inaequalis* and *Venturia pirina*. *J. gen. Microbiol.* 17, 120.

ROACH, W. A. (1934). Effect of sodium thiosulphate on apple mildew. *Ann. appl. Biol.* 21, 341.

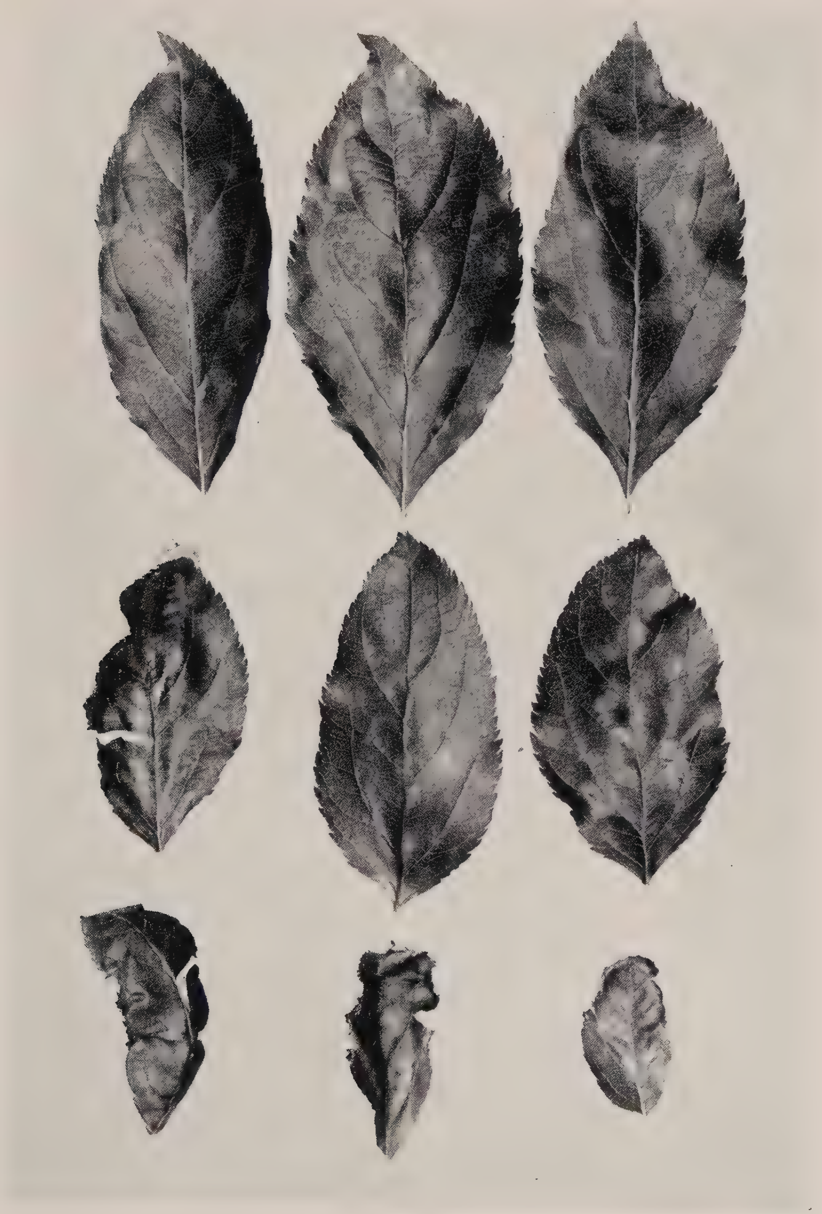
ROACH, W. A. (1938). Plant injection for diagnostic and curative purposes. *Tech. Commun. Bur. Hort., E. Malling* no. 10.

WINKELMANN, A., HOLZ, W. O. & JAENICHEN, H. (1937). Beiträge zur Biologie und Bekämpfung des Apfelschorfes (*Fusicladium dendriticum* (Wallr.) Fckl.). *III. Mitt. Zbl. Bakt.* (2 *Abt.*), 96, 177.

EXPLANATION OF PLATE

Fig. 1. Damage to leaves of Cox's Orange Pippin caused by injection of chlorogenic acid and polyphenolic metabolites of Williams' pear (× $\frac{1}{3}$). Left to right: leaves -1, L_0 and $+1$. Upper: water control; middle: chlorogenic acid; lower: Williams 3 extract.

(*Received* 17 *April* 1957)



D. S. KIRKHAM—HOST RELATIONS OF *VENTURIA* SPP. PLATE 1

(*Facing p.* 504)

THORNE, C. B. & BELTON, F. C. (1957). *J. gen. Microbiol.* 17, 505–516

An Agar-Diffusion Method for Titrating *Bacillus anthracis* Immunizing Antigen and its Application to a Study of Antigen Production

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SUMMARY: An agar-diffusion method is described for the titration of *Bacillus anthracis* immunizing antigen and antibody. It is a sensitive and simple method that can be used for determining antigen concentrations in culture filtrates and for titrating antisera from animals and humans which have been immunized with the antigen. Marked improvements in yields of antigen in a defined medium and a hydrolysed casein + amino acids (Casamino) medium have been achieved with the aid of this assay method.

Although much information has been gained from studies on the protective antigen present in culture filtrates of *Bacillus anthracis* when the organism is grown under certain conditions (Gladstone, 1946, 1948; Wright, Hedberg & Slein, 1954; Puziss & Wright, 1954; Belton & Strange, 1954; Strange & Belton, 1954), research on this antigen has been hampered by the lack of convenient assay methods for the antigen and its antibody. Recently Belton & Henderson (1956) developed a skin test in rabbits for assaying the immunizing antigen and antibody, based on the capacity of antiserum to neutralize anthrax toxin. This method proved to be valuable for assaying antigen preparations and particularly useful in titrating immune sera. The present paper describes another assay procedure, an agar-diffusion method based on the technique of Ouchterlony (1953), which is much simpler and more sensitive than the rabbit skin test. We have used the new assay method with good results in a study of some factors which affect production of antigen. Studies by Wright *et al.* (1954), Puziss & Wright (1954) and Belton & Strange (1954) resulted in methods for producing immunizing antigen in protein-free media. The present investigation was undertaken to improve yields of antigen in order to facilitate research on the more fundamental aspects of its biochemistry.

METHODS

Organisms. Most of the work on antigen production was done with the avirulent Weybridge strain isolated by Sterne (1937) and used by the Ministry of Agriculture, Fisheries and Food as a living spore vaccine. A few experiments were done with the following strains: Vollum; N.P.A., a virulent non-proteolytic mutant from Vollum; M-36; BI, an avirulent strain. Spore suspensions were prepared as described by Belton & Strange (1954).

Media and methods for antigen production. The defined medium was the 599 medium described by Puziss & Wright (1954) for antigen production. It contained 14 amino acids, guanine, thiamine, $CaCl_2$, $MgSO_4$, $MnSO_4$, $FeSO_4$, K_2HPO_4, KH_2PO_4, $NaHCO_3$, and glucose. The 'Casamino acids' medium, which was developed by Belton & Strange (1954) for antigen production, contained Difco Casamino Acids, glycine, tryptophan, cystine, glutamine, adenine, uracil, guanine, thiamine, glucose, $NaHCO_3$, salts as in 599 medium, and charcoal. Media were prepared with distilled water which had been passed through an Amberlite resin (Belton & Strange, 1954) and were autoclaved at pH 6·9 for 15–20 min. at 15 lb./sq.in. Glutamine, glucose, $NaHCO_3$, and $FeSO_4$, when used at the original concentration of 0·0005 M, were sterilized by filtration. When the concentration of $FeSO_4$ was 0·000025 M it was autoclaved in the medium.

Cultures were grown at 37° either in 3 l. Thompson bottles containing 500 ml. medium or in 250 ml. Erlenmeyer flasks containing 100 ml. The inoculum was either 10^6 spores or 0·1 ml. of a 24 hr. shaken culture grown in the defined medium without $NaHCO_3$. Thompson bottles were incubated statically and flasks were incubated either statically or on a reciprocating shaker with a stroke distance of 2·5 cm. and a speed of 110 cycles/min. The time of incubation varied and is given for individual experiments.

Preparation of samples for assay. Cultures were filtered through sintered glass and usually 10 ml. samples of filtrates were lyophilized with 0·5 ml. of normal horse serum. This was a convenient procedure since serum stabilized the antigen to lyophilization and such samples could be stored for later reference. At other times samples were assayed directly after adding 5% (v/v) horse serum. In experiments testing the effect of gelatin on the stability of antigen to lyophilization 10% (v/v) of a 2% (w/v) solution in 0·02 M-phosphate (pH 8·0) was added. For testing the effect of gelatin on the assay method either 5% (v/v) of the gelatin solution was added directly to the sample before diluting it in saline or dilutions were made in 0·5% (w/v) gelatin in 0·02 M-phosphate (pH 8·0). Potencies were estimated by the agar diffusion method and improvements in yields were confirmed by the toxin neutralization test (Belton & Henderson, 1956) and/or immunization tests in rabbits.

Immunization tests. Rabbits of heterogeneous strains weighing about 5 lb. were used. Five doses of 0·5 ml. each of appropriately diluted samples of antigen were given subcutaneously at 2-day intervals and the animals were challenged with an intradermal injection of 500 spores (250 lethal doses) of *Bacillus anthracis* strain M-36, 7 days after the last dose of antigen. Results are reported as survival ratios, i.e. the number of animals surviving divided by the number challenged.

Medium for agar diffusion plates. 1% agar dissolved in 0·075 M-phosphate buffer (pH 7·3) containing 0·9% NaCl and 0·01% thiomersalate.

Antisera. Antiserum H-533 prepared in a horse by injecting Weybridge spores was used routinely. Antiserum H-25 prepared in a horse by injecting alum precipitated antigen (Belton & Strange, 1954) was used in some of the tests. In addition two samples of hyperimmune spore antisera produced in horses for commercial use were supplied by Dr H. N. Spears (Ministry of

Agriculture, Fisheries and Food Laboratories, Weybridge). One of these had been lyophilized and stored for two years.

Standard antigen. A large batch of culture filtrate from the Weybridge strain grown in the improved Casamino acids medium was lyophilized in 10 ml. portions with 0·5 ml. of normal horse serum. This preparation, S-172, which had a titre of 1/19·2 in the agar diffusion assay with the standard antiserum H-533, was used as a standard antigen for antibody titrations and for comparing line patterns of other antigen preparations.

Titration of antigen. Twelve ml. of the agar medium were used in each Petri dish (9 cm. diam.) and circular reservoirs were made in the solidified agar with cork borers. Plates prepared in this manner were stored at 2° and used as required. Several sizes and arrangements of reservoirs were tested and best results were obtained with a plan designed by Dr T. W. Burrows of this department (Pl. 1, figs. 1, 2). A pattern with holes to accommodate cork borers of the appropriate sizes was made of Perspex to fit over a Petri dish. The holes in the two outer rows are 7 mm. in diameter and are spaced 5 mm. apart and 6 mm. from the inner row of holes which are 5 mm. in diameter and 7 mm. apart.

The inner reservoirs were filled with undiluted antiserum and the plates were held at 2° for 18–24 hr. The outer reservoirs were then filled with appropriate dilutions of antigen samples in saline (or in 0·5 %, w/v) gelatin in 0·02 M-phosphate (pH 8·0, when horse serum or gelatin had not been added to the sample), after which the plates were kept at room temperature. Plates were usually observed after 20–24 hr. for lines of precipitation by holding over a light against a black background, but final readings were not made until the second day. The highest dilution which produced a visible line was taken as the end point.

As a first approximation of potency usually five successive twofold dilutions of each of two samples were put on a single plate. After the potency had been determined in this manner it was possible to do a closer titration by diluting samples over a twofold range in five steps differing by dilution factors of 10/12, 10/14, 10/16, 10/18 and 10/20. With these close dilution factors the end point was still clearly visible and reproducible.

Titration of antibody. Antibody was titrated by determining the capacity of test antiserum to combine with standard antigen and to prevent its forming a line of precipitation with a standard antiserum in agar diffusion plates. The concentration of antigen in a 1/8 final dilution of the standard preparation, S-172, was found convenient and suitable for antibody assays. Samples, usually 0·2 ml., of the standard antigen diluted 1/4 in saline, were mixed with equal volumes of serial twofold dilutions of the test antiserum. The resulting solutions were added directly to antigen reservoirs in agar plates prepared as described for antigen titrations with standard antiserum H-533 in the centre reservoirs. The end-point was taken as the highest final dilution of test antiserum that prevented formation of a visible line.

RESULTS

Antigen titrations

Pl. 1, fig. 1, shows a typical plate with two samples assayed against antiserum H-533. In the upper part of the plate the five reservoirs contained a lyophilized filtrate from the Weybridge strain at dilutions of 1, 1/2, 1/4, 1/8 and 1/16 respectively from left to right. The line of precipitation did not extend beyond the fourth reservoir and therefore the titre was 1/8 < 1/16. The reservoirs in the lower half of the plate contained a lyophilized filtrate from the Vollum strain reconstituted to 2, 1, 1/2, 1/4 and 1/8 times the original concentration. The titre of this sample was 1/2 < 1/4.

Since the titre of a sample depends not only on the concentration of antigen but also on the strength of the antiserum used, the test antiserum must be identified when potencies are expressed. For example, with a sample of partially purified antigen the end-point was reached at a concentration of about 4 μg./ml. in titrations against antiserum H-533 and about 8 μg./ml. in titrations against antiserum H-25.

Reproducibility of results. Table 1 shows results obtained when each of four lyophilized culture filtrates was assayed four times. The data demonstrate that results were highly reproducible over a period of time.

Table 1. *Reproducibility of antigen titrations by the agar diffusion method*

Sample no.	Titre obtained against antiserum H-533 on given date			
	5 Nov.	8 Nov.	30 Nov.	1 Mar.
1	1/2 < 1/4	1/2 < 1/2·4	1/2 < 1/2·4	1/2 < 1/2·4
2	1/4 < 1/8	1/5·6 < 1/6·4	1/5·6 < 1/6·4	1/4·8 < 1/5·6
3	1/8 < 1/16	1/9·6 < 1/11·6	1/9·6 < 1·11/2	1/9·6 < 1/11·2
4	1/8 < 1/16	1/14·4 < 1/16	1/12·8 < 1/14·4	1/12·8 < 1/14·4

Effect of normal serum and gelatin. The addition of normal horse serum to samples (see under *Preparation of samples for assay*) served two purposes; it stabilized the antigen to lyophilization and increased the sensitivity of the agar diffusion method about twofold. This effect of serum on the assay was observed repeatedly with a purified antigen preparation and culture filtrates. The effect was not a specific one of serum, however, since the same results with the assay method and in the protection of antigen to lyophilization were obtained when gelatin was substituted for serum.

Results with other antisera. The two hyperimmune sera obtained from the Ministry of Agriculture, Fisheries and Food (Weybridge Laboratories) were used to assay several samples of antigen which were also titrated against antiserum H-533. Results obtained with the three antisera were identical.

Correlation of results with animal tests. Before one could rely on the agar diffusion assay as a method for estimating actual immunizing potencies, it was necessary to test several samples both by the new method and by animal protection tests. Since it had been shown earlier that results of the rabbit skin test for anthrax toxin neutralization (Belton & Henderson, 1956) closely paralleled results of animal protection tests, this method of testing potencies was included. Results of testing five samples by the three methods are shown in Table 2. Potencies as determined by the skin test and the agar diffusion method agreed closely and the relative potencies were confirmed by the immunization tests. These results, as well as others from later experiments, indicate that the agar diffusion assay is reliable for titrating antigen.

Table 2. *Comparison of three methods for estimating potencies of antigen samples*

Culture filtrate no.	Medium*	Highest dilution active in rabbit skin test	Titre against antiserum H-533 in agar diffusion plates	Immunizing activity for rabbits: survival ratios† at given dilutions				
				Undiluted	1/5	1/15	1/25	1/50
1	Casamino acids medium of Belton & Strange (1954) without charcoal	1<1/2	1/1·4<1/1·6	4/4	3/4	1/4	.	.
2	Same as 1 with 0·1% (w/v) charcoal	1/2<1/4	1/3·2<1/3·6	.	4/4	2/4	1/4	.
3	Same as 1 with 0·1% (w/v) charcoal and 5% (v/v) sheep serum	1/8<1/16	1/6·4<1/7·2	.	4/4	.	4/4	3/4
4	599 medium of Puziss & Wright (1954) with 0·05% (w/v) charcoal	1/2<1/4	1/1·6<1/1·8	.	2/4	2/4	1/4	.
5	Same as 4 with 5% (v/v) horse serum	1/8<1/16	1/11·2<1/12·8	.	4/4	.	4·4	3/4

* The Weybridge strain was grown in Thompson bottles. Those with Casamino acids medium were incubated 22 hr. and those with defined medium were incubated 40 hr.

† Survival ratios of rabbits immunized with given dilutions of antigen and challenged by intradermal injection of spores.

Multiple lines of precipitation. Samples taken from static cultures at or before the time of maximum antigen yield formed only one line of precipitation when tested at filtrate concentration or after diluting. More concentrated samples of such filtrates, as well as diluted samples of filtrates prepared under various other conditions, often formed more than one line. This was true with filtrates from older cultures and particularly so with samples from shaken cultures in which antigen formation and destruction both occurred more rapidly than in static cultures.

Pl. 1, fig. 2, shows lines formed by four representative filtrates, described in Table 3, when tested at two concentrations. The upper row of reservoirs contained the samples at filtrate concentration and the lower row contained the same samples concentrated fourfold. The main line formed by the standard antigen preparation S-172 merged with the main line formed by the other filtrates, with the exception of sample 300, the line of which crossed the line of S-172. Data in Table 3 show that all samples except 300 had immunizing activities correlating with titres as determined by the agar diffusion method.

Table 3. *Description of filtrates tested on agar diffusion plate shown in Fig.* 2

Filtrate no.	Cultural medium and conditions*	Titre against anti-serum H-533	Highest dilution active in rabbit skin test	Immunizing activity for rabbits: survival ratios at given dilutions						
				Undiluted	1/5	1/15	1/30	1/45	1/60	1/180
S-172	Modified Casamino acids medium incubated statically 22 hr. (standard antigen preparation used for antibody titrations and for comparing lines formed by other preparations)	$1/19{\cdot}2 < 1/22{\cdot}4$	Not tested	.	.	.	4/4	.	4/4	0/4
301	Modified Casamino acids medium in shaken flasks incubated 16 hr.	$1/8 < 1/16$	$1/8 < 1/16$	4/4	4/4	3/4	.	2/4	.	.
300	Modified Casamino acids medium with concentration of amino acids doubled, in shaken flasks incubated 94 hr.	$1/19{\cdot}2 < 1/22{\cdot}4$	Undiluted	1/4	.	0/4	.	.	.	.
217	Synthetic medium 599 with concentration of $FeSO_4$ decreased to 0·000025 M, 0·6 % $NaHCO_3$, incubated statically 40 hr.	$1/8 < 1/9{\cdot}6$	$1/8 < 1/16$	.	.	5/8	4/7	.	1/7	.

* The Weybridge strain was used for all samples.

This correlation of line patterns with immunizing activity was observed repeatedly. Of a number of samples tested those that formed a line which merged with the main line of S-172 had immunizing activity. These observations demonstrate that the antigen which produced the line given by sample 300 had insignificant protective activity and they provide evidence that the antigen responsible for the main line formed by the standard preparation S-172 represented a protective antigen. As many as five lines have been observed with highly concentrated filtrates or filtrate from old cultures. The nature of the antigens which form the various lines has not yet been ascertained. Possibilities to be considered are that some of them represent other protective antigens, precursors or degraded products of a single protective antigen, or antigens not related to the protective antigen.

Antibody titrations

Each of 250 human sera was titrated by the agar diffusion method and by the rabbit skin test for toxin neutralization; the titres obtained by the two methods agreed closely. Table 4 presents results obtained when ten representative antisera were titrated three times by the agar diffusion method. The data show that results were reproducible and that they agreed closely with results from the skin test. This new method has also been used to titrate antisera from rabbits, guinea pigs and horses, including some with titres as high as 1/1600.

Table 4. *Reproducibility of antibody titrations*

Antiserum no.	Titre by toxin neutralization test	Titre by agar diffusion method		
		First test	Second test	Third test
1	1/8	1/4	1/4	1/4
2	1/8	1/8	1/8	1/8
3	1/4	1/4	1/4	1/2
4	1/8	1/8	1/4	1/4
5	1/16	1/16	1/16	1/16
6	1/2	1/4	1/2	1/2
7	Nil	Nil	Nil	Nil
8	1/4	1/4	1/4	1/4
9	1/16	1/16	1/32	1/16
10	1/16	1/16	1/16	1/16
Normal serum	Nil	Nil	Nil	Nil

Studies on antigen production

Definied medium for growth. When the defined medium was tested with the Weybridge strain in flasks or Thompson bottles, filtrates had antigen titres of 1 or ½ against antiserum H-533. The yield of antigen was increased at least eightfold by making the modifications in the medium described below.

A decrease in the concentration of $FeSO_4$ resulted in a higher yield of antigen; the yield was doubled when the concentration was reduced from 0·0005 to 0·000025 M. In experiments with a vegetative inoculum it was shown that $FeSO_4$ need not be omitted from the medium, although traces were introduced with the inoculum since it was grown in the defined medium containing 0·000025 M-$FeSO_4$. However, when a spore inoculum was used and $FeSO_4$ not added, the organism grew very slowly, as had been shown by Puziss & Wright (1954).

Belton & Strange (1954) obtained higher yields of antigen by adding charcoal to the Casamino acids medium. We have found that charcoal has a similar effect in the defined medium. The reason for this effect of charcoal is not clear. It was also shown previously that to be effective the charcoal had to be present in the medium during growth; this was confirmed in the defined medium. When charcoal was autoclaved with the medium and then removed by filtration or when it was added aseptically to the culture at 24 hr. there was no effect on antigen yields.

The effects of various concentrations of $NaHCO_3$ on antigen yields are shown in Table 5. Without $NaHCO_3$ no detectable antigen was produced. This is in agreement with results obtained by Gladstone (1946) and Puziss & Wright (1954). When the concentration of $NaHCO_3$ was increased to 0·5 or 0·75% (w/v) the antigen yields increased about fourfold, but a concentration of 1·0% (w/v) was greater than the optimum concentration. From these results and those of similar experiments a concentration of 0·6% (w/v) was selected for routine use. With the increased concentration of $NaHCO_3$ in the defined medium it was necessary to use a vegetative inoculum. Poor growth and low antigen yields resulted when spore inocula were used.

Table 5. *Effect of* $NaHCO_3$ *on antigen yields in the medium**

$NaHCO_0$ % (w/v)	pH value of filtrate		Titre of filtrate: against antiserum H-533	
	22 hr.	40 hr.	22 hr.	40 hr.
0	.	7·0	<1	<1
0·25	.	8·3	<1	$1/2<1/4$
0·50	8·6	8·5	$1/2<1/4$	$1/8<1/16$
0·75	8·8	8·8	$1/2<1/4$	$1/8<1/16$
1·0	9·0	9·0	$1<1/2$	$1/4<1/8$

* 599 medium with 0·000025 M-$FeSO_4$; 100 ml. in 250 ml. Erlenmeyer flasks incubated statically; vegetative inoculum, Weybridge strain; pH 6·9 before addition of $NaHCO_3$.

A few experiments were done on the effect of replacing $NaHCO_3$ with Tris (tris-(hydroxymethyl)-aminomethane) buffer. Without added $NaHCO_3$ antigen was produced when 0·05 M-Tris was used at a sufficiently high initial pH value, i.e. 8·7–9·5, although the yields with Tris were lower than those with $NaHCO_3$. These results suggested that bicarbonate may not be essential for antigen synthesis but that its function in the medium may be to provide and maintain a favourable pH value. However, with Tris buffer at a high pH value enough CO_2 might have been retained from that produced by the organism to supply sufficient bicarbonate for antigen synthesis.

Table 6 shows the yields of antigen obtained with various modifications of

Table 6. *Improved yields of antigen in defined medium**

Sample no.	Medium	Titre against antiserum H-533	Immunizing activity for rabbits: survival ratios at given dilutions				
			Undiluted	1/5	1/15	1/30	1/60
1	599 medium	$1/1{\cdot}8 < 1/2$	7/8	4/7	2/8	.	.
2	599 medium with concentration of $FeSO_4$ decreased to 0·000025 M	$1/3{\cdot}6 < 1/4$	Not tested				
3	Same as 2 with concentration of $NaHCO_3$ increased to 0·6 %	$1/8 < 1/9{\cdot}6$	.	.	5/8	4/7	1/7
4	599 medium with 0·000025 M-$FeSO_4$, 0·005 M-DL-alanine and 0·002 M-glycine added, concentration of glutamic and aspartic acids increased fivefold, 0·6 % $NaHCO_3$	$1/11{\cdot}2 < 1/12{\cdot}8$	.	.	6/8	6/8	1/8
5	Same as 4 with 0·05 % charcoal	$1/14{\cdot}4 < 1/16$	.	.	8/8	5/8	4/7
6	599 medium with 0·000025 M-$FeSO_4$, concentrations of all amino acids doubled, 0·005 M-DL-alanine and 0·002 M-glycine added, 0·6 % $NaHCO_3$	$1/8 < 1/16$	Not tested				
7	Same as 6 with 0·05 % charcoal	$1/19{\cdot}2 < 1/22{\cdot}4$	Not tested				

* 100 ml. medium in 250 ml. Erlenmeyer flasks incubated statically 40 hr.; vegetative inoculum, Weybridge strain.

the defined medium and gives the results of some protective tests in rabbits. With the increased concentration of $NaHCO_3$ and the decreased concentration of $FeSO_4$ yields were improved further when the medium was enriched with alanine and glycine and when the concentrations of other amino acids were increased. Maximum yields occurred at 32–40 hr. The results of rabbit protection tests confirmed the improved yields in those samples which were tested.

Casamino acids medium. Yields of antigen from static cultures grown in Casamino acids medium were increased several-fold when $FeSO_4$ was not added and the concentration of $NaHCO_3$ increased to 0·5 or 0·6% (w/v). Additional studies revealed that the medium could be simplified by omitting glutamine and guanine, and yields of antigen were improved further by replacing the potassium phosphate by 424 mg. KCl/l. (It should be pointed out that Difco casamino acids contain small amounts of iron and phosphate.) Table 7 shows yields that were obtained with the original and modified media. The increase of yields of antigen as determined by the agar diffusion method of assay was confirmed by animal tests.

When other strains of *Bacillus anthracis*, namely BI, Vollum, N.P.A. and M-36, were tested, yields of antigen were 4–8 times higher in the modified casamino acids medium than in the original medium.

Shaken flask cultures. When the modified Casamino acids medium (as given above except with the original amount of phosphate buffer) was tested in shaken flasks, interesting results were obtained. An antigen titre of 1/8 against antiserum H-533 was produced in 16–18 hr. and it decreased very rapidly during the next 8 hr. With the decrease in antigen titre, as determined by the agar diffusion method as well as by animal tests, interesting changes in the pattern of lines occurred. When the titre was at its peak usually only one line was observed and as the titre decreased multiple lines appeared. The results suggested that the antigen was perhaps being degraded into smaller fragments.

When a richer medium prepared by doubling the concentration of casamino acids was tested in shaken flasks filtrates of 14–20 hr. cultures formed a line which merged with that of the standard preparation, S-172. After 20 hr. this antigen disappeared rapidly and antigen forming a different line appeared as shown by sample 300 in Fig. 2. Although the titres of this antigen were as high as 1/16 to 1/32, such filtrates had insignificant activity in toxin neutralization and immunization tests.

Conclusion

The agar diffusion method is a simple and inexpensive assay procedure for the protective antigen of *Bacillus anthracis*. Being sensitive enough to detect relatively small changes in antigen concentration, it should be useful in studies on fundamental aspects of the organism's physiology relating to antigen synthesis. The method, as well as the increased antigen yields resulting from the present study, should be valuable in research on isolation and identification of the active principle. The method for titrating antibody is a simple one that should be useful in anthrax immunity studies.

Table 7. *Improved yields of antigen in Casamino acids media*

Sample no.	Medium*	Titre against antiserum H-533	Immunizing activity for rabbits: survival ratios at given dilutions					
			1/5	1/15	1/25	1/30	1/60	1/180
1	Original Casamino acids medium of Belton & Strange (1954)	$1/2 < 1/4$	4/4	2/4	1/4	.	.	.
2	Same as 1 with $FeSO_4$ omitted	$1/9{\cdot}6 < 1/11{\cdot}2$	.	4/4	.	2/4	1/4	.
3	Same as 1 with $FeSO_4$, guanine, glutamine and phosphate omitted, 424 mg. KCl/l. added, concentration of $NaHCO_3$ increased to 0·6 %	$1/16 < 1/19{\cdot}2$	Not tested					
4	Same as 3	$1/19{\cdot}2 < 1/22{\cdot}4$	.	.	.	4/4	4/4	0/4†

* The Weybridge strain was used and all cultures were grown in Thompson bottles for 22 hr.

† Alum-precipitated antigen (Belton & Strange, 1954) prepared from the original Casamino acids medium afforded protection to 50 % of rabbits tested at a dilution of 1/15, based on original filtrate concentration. Similar preparations from the modified Casamino acids medium protected 50 % at a dilution of 1/180.

It is a pleasure to acknowledge the helpful suggestions of Dr D. W. Henderson. We are also grateful to Dr H. N. Spears of the Ministry of Agriculture, Fisheries and Food (Weybridge Laboratories) for two samples of hyperimmune horse sera. This work was done during a year's working visit by one of us (C. B. T.) from Fort Detrick, Frederick, Maryland, U.S.A.

REFERENCES

Belton, F. C. & Henderson, D. W. (1956). A method for assaying anthrax immunising antigen and antibody. *Brit. J. exp. Path.* **37**, 156.

Belton, F. C. & Strange, R. E. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: medium and methods of production. *Brit. J. exp. Path.* **35**, 144.

Gladstone, G. P. (1946). Immunity to anthrax: protective antigen present in cell-free culture filtrates. *Brit. J. exp. Path.* **27**, 394.

Gladstone, G. P. (1948). Immunity to anthrax: production of the cell-free protective antigen in cellophane sacs. *Brit. J. exp. Path.* **29**, 379.

Ouchterlony, O. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in co-ordinated systems of diffusion. *Acta path. microbiol. Scand.* **32**, 231.

Puziss, M. & Wright, G. G. (1954). Studies on immunity in anthrax. IV. Factors influencing elaboration of the protective antigen of Bacillus anthracis in chemically defined media. *J. Bact.* **68**, 474.

Sterne, M. (1937). Variation in *Bacillus anthracis*. *Onderstepoort J. vet. Sci.* **8**, 271.

Strange, R. E. & Belton, F. C. (1954). Studies on a protective antigen produced *in vitro* from *Bacillus anthracis*: purification and chemistry of the antigen. *Brit. J. exp. Path.* **35**, 153.

Wright, G. G., Hedberg, M. A. & Slein, J. B. (1954). Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically-defined non-protein medium. *J. Immunol.* **72**, 263.

EXPLANATION OF PLATE

Fig. 1. Agar diffusion plate demonstrating titrations of antigen in two culture filtrates.

Fig. 2. Agar diffusion plate showing line patterns formed by four different culture filtrates at two concentrations.

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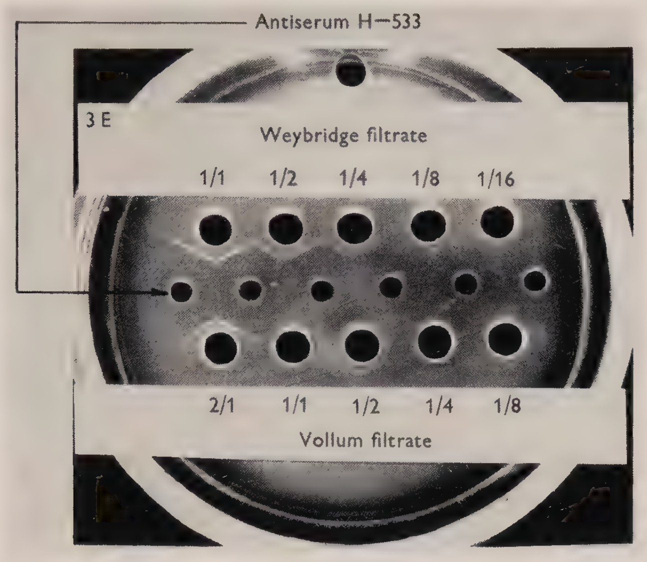
Antiserum H–533
3 E
Weybridge filtrate
1/1
1/2
1/4
1/8
1/16
2/1
1/1
1/2
1/4
1/8
Vollum filtrate

Fig. 1

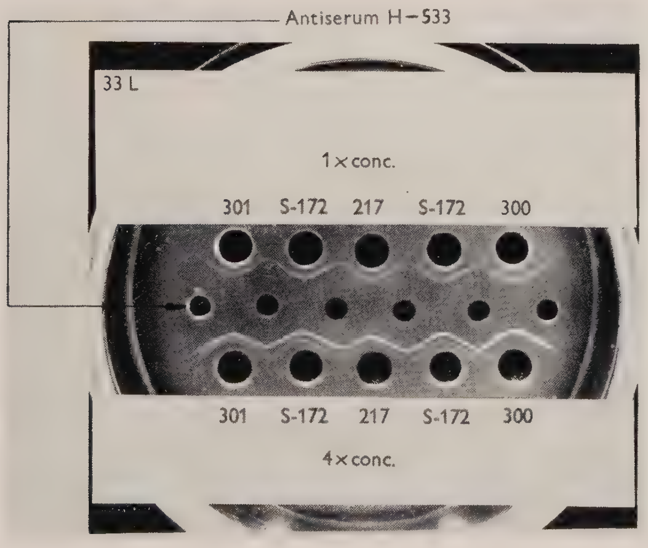
Antiserum H–533
33 L
1 × conc.
301
S-172
217
S-172
300
301
S-172
217
S-172
300
4 × conc.

Fig 2

C. B. Thorne & F. C. Belton—Anthrax antigen assay. Plate 1

Pereira, H. G. & Kelly, B. (1957). *J. gen. Microbiol.* **17**, 517–524

Dose-response Curves of Toxic and Infective Actions of Adenovirus in HeLa Cell Cultures

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SUMMARY: Study of the dose-response curves in titrations of adenovirus type 5 in cultures of HeLa cells suggests two different mechanisms of cytopathic effect. A late effect, caused by relatively small virus doses, is considered to be a manifestation of virus infectivity and is in good agreement with the hypothesis of independent action of virus units in the initiation of infection. The early effect, on the other hand, requires large virus doses and is considered to be of a toxic nature. Infective and toxic properties of adenovirus type 5 are distinguished by the greater sensitivity of the latter to inactivation by ultraviolet light.

The methods used for infectivity titrations of adenoviruses are based on the cytopathic action of these agents in tissue cultures. Titration techniques described by different workers vary in several respects but in most cases the results are based on quantal responses in cultures incubated for a given period. A relationship between virus dose and time of onset of cytopathic effect has been described (Rowe, Huebner, Gilmore, Parrott & Ward, 1953), and Kjellen (1956) developed a method of titration based on the latent period which precedes the cytopathic effect.

In the course of repeated titrations of adenovirus preparations we observed a difference between the slopes of dose-response curves of titrations read after varying periods of incubation. These differences suggest that the early effect of adenoviruses in HeLa cell cultures is of the nature of a toxic action, whereas the late effect is a manifestation of the infective property of the virus resulting from the independent action of single infective units.

Studies on the dose-response curves of bacterial and virus titrations have been extensively reviewed and discussed (Beard, Sharp & Eckert, 1955; Bryan, 1955; Isaacs, 1957; Lauffer & Price, 1945; Luria, 1940; Meynell & Stocker, 1957). In most infectivity titrations the log dose-probit response curves show a slope equal to or lower than 2 (Peto, 1953), this being approximately the slope of the first term of the Poisson distribution (the 'one-particle curve'). This finding is generally accepted as evidence for the hypothesis of independent action of infective units, a single unit being sufficient to initiate infection. Deviations from the one-particle curve are attributed to variations in host susceptibility which cause a flattening of the curves. This is particularly evident with agents such as the tumour viruses where variation in host susceptibility greatly affects the slope of dose-response curves (Bryan, 1954; Beard *et al.* 1955). With bacteriophages, on the other hand, where host susceptibility is uniform, agreement with the one-particle curve is good (Feemster & Wells, 1933; Luria,

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1939). In the study of animal viruses host variation may be diminished by using tissue culture techniques and results agreeing with the hypothesis of independent action of virus units have been reported in titrations based on quantal responses (Sheffield & Smith, 1956) and on plaque counts (Dulbecco, 1952).

The cytopathic effect of viruses in tissue cultures is in most cases a consequence of virus multiplication resulting in structural alterations of the host cell. It has, however, been demonstrated by Henle, Girardi & Henle (1955) that influenza virus may cause a cytopathic effect not associated with reproduction of infective virus. This effect requires a large multiplicity of infection and probably depends on the synergistic action of virus particles. It is therefore to be expected that dose-response curves of titrations based on this cytotoxic effect in cultures of relatively uniform susceptibility should give a slope steeper than that of the one-particle curve. Our suggestion of a toxic nature for the early effect of adenovirus in HeLa cells was based on this reasoning. A further distinction between early and late effects observed in our titrations was provided by the greater resistance of the former to inactivation by ultraviolet light. Similar findings have been reported in connexion with the toxic effect of influenza viruses in animals (Henle, G. & Henle, W., 1946; Henle, W. & Henle, G., 1946, 1947) and in tissue cultures (Henle *et al.* 1955).

METHODS

HeLa cell cultures were propagated and maintained in the following media: medium I: 10% (v/v) human serum, 0·5% (w/v); lactalbumin hydrolysate (Nutritional Biochemical Corporation, Cleveland, Ohio); 0·1% (w/v) yeast extract (Difco) in balanced saline solution (Gey & Gey, 1936). Medium II: 5% (v/v) normal rabbit serum; 0·5% (w/v) lactalbumin hydrolysate in balanced saline solution. Medium III: 5% (v/v) normal rabbit serum; 0·25% (w/v) lactalbumin hydrolysate in balanced solution. Penicillin (100 units/ml.), streptomycin (100 mg./ml.) and mycostatin (20 units/ml.) were added to all media. The balanced saline solution was prepared according to the formula given by Gey & Gey (1936) except that the concentration of sodium bicarbonate was decreased to 1·125 g./1000 ml. Human serum was used in pools of three or four samples, filtered through GS sterimats and stored at −15°. Rabbit serum was obtained from adult Dutch rabbits bled by heart puncture under ether anaesthesia. The serum was separated by centrifugation, heated at 60° for 30 min. and stored at −15°. Lactalbumin hydrolysate was used as a 5% (w/v) stock solution in calcium- and magnesium-free balanced saline solution, autoclaved at 8–9 lb./sq.in. for 10 min. A 1% (w/v) stock solution of yeast extract was prepared similarly.

Stock HeLa cell cultures were grown in 250 ml. bottles incubated in a roller drum at 37°. Bottles containing about 10^7 cells were each subcultured either to three new bottles or to 100 tubes by the following technique: the fluid medium was removed as completely as possible and 2·5 ml. of a 0·5% (w/v) solution of trypsin (L. Light and Co., Colnbrook, Bucks.) were added to each bottle. After a 10 min. incubation at 37° most of the cells were pipetted off and transferred to a centrifuge tube. Each bottle was rinsed with 5 ml. balanced saline solution which was pooled with the cell suspension. Cellular clumps were broken up by vigorous pipetting and the suspension was centrifuged for 5 min. at about 100 rev./min. After removal of the supernatant fluid, the cells from each bottle were resuspended either in 30 ml. medium I or in 50 ml. medium II to be transferred to three new bottles or to 100 tubes respectively. The cell suspension was dispensed into tubes ($3 \times \frac{5}{8}$ in. Pyrex tubes) by means of an automatic syringe connected to a reservoir in which the cells were kept in suspension by a magnetic stirrer. Tubes were ready for use after overnight incubation at 37°, without rotation, at an angle of 5° from the horizontal. Each tube then received 0·4 ml. of a 5 % (v/v) dilution of normal rabbit serum in balanced saline solution followed by 0·1 ml. of the virus inoculum. The volume of medium/tube was thus made up to 1 ml. and the final concentration of lactalbumin hydrolysate was decreased to 0·25 % (w/v). The tubes were re-incubated at 37° and at intervals of 6 or 7 days the fluid from each tube was removed and replaced by 1 ml. of fresh medium III.

Virus preparation. The prototype strain of adenovirus type 5 was received from Dr R. J. Huebner (National Institutes of Health, Bethesda, Maryland, U.S.A.) and propagated in our laboratory through four passages in HeLa cell cultures. The material used throughout this work was obtained from two bottles containing each approximately 10^7 HeLa cells and 10 ml. of medium III, inoculated with $10^{6\cdot5}$ 50 % virus infective doses. After 4 days of incubation the total contents of the bottles were harvested and centrifuged for 10 min. at 2000 rev./min. Nine-tenths of the supernatant fluid were decanted and the remaining volume, together with the cellular deposit, was ground in a Ten-Broeck tissue grinder and again centrifuged for 10 min. at 2000 rev./min. The two supernatant fluids were pooled, divided into 1 ml. volumes and stored at −15°.

Virus titrations. Serial dilutions of the virus preparation were made in broth saline (10 %, v/v, tryptic digest broth in normal saline), using a fresh pipette for each dilution. Volumes of 0·1 ml. of each dilution were added to each of thirty-two tubes of HeLa cells. The cultures were examined microscopically at suitable intervals for evidence of cytopathic effect. Tubes showing non-specific degeneration, usually due to incubation at an incorrect position or to loosening of bungs, were discarded.

RESULTS

Dose-response curves

Three titrations of the virus preparation were carried out at dilution ranges expected to give cytopathic effect end-points at 3, 9 and 20 days, respectively. The results of these titrations are shown in Table 1. The goodness of fit with the first and second terms of the Poisson distribution were estimated by the χ^2 test according to the method recommended by Haldane (1939). The data were also submitted to probit analysis and the log dose-probit response curves

Table 1. *Adenovirus titrations read after varying periods of incubation*

Titration no.	Period of incubation (days)	Virus dilution*	a	b	b′	b″	Slope of log-probit curve
1	3	$10^{-4\cdot2}$	29	1	22·5	12·2	$6\cdot25 \pm 1\cdot03$
		$10^{-4\cdot4}$	19	11	25·2	19·4	
		$10^{-4\cdot6}$	2	28	26·9	24·5	
		$10^{-4\cdot8}$	1	24	23·4	22·9	
					$\chi=126\cdot5$	28·6	
					$P<0\cdot001$	$P<0\cdot001$	
2	9	$10^{-7\cdot6}$	30	2	1·5	0·4	$2\cdot47 \pm 0\cdot33$
		$10^{-7\cdot9}$	27	4	6·8	5·4	
		$10^{-8\cdot2}$	19	11	14·0	15·9	
		$10^{-8\cdot5}$	10	22	21·9	25·9	
		$10^{-8\cdot8}$	3	29	26·5	30·1	
					$\chi^2=4\cdot2$	13·7	
					$P>0\cdot3$	$P<0\cdot01$	
3	20	$10^{-8\cdot8}$	31	1	1·0	0·1	$2\cdot11 \pm 0\cdot33$
		$10^{-9\cdot1}$	25	3	4·8	2·8	
		$10^{-9\cdot4}$	22	10	13·3	13·6	
		$10^{-9\cdot7}$	10	22	20·6	23·9	
		$10^{-10\cdot0}$	10	22	25·7	29·4	
					$\chi^2=9\cdot0$	34·4	
					$P>0\cdot05$	$P<0\cdot001$	

* Final dilution in 1 ml. of medium.

a = number of positive cultures; b = number of negative cultures; b′ = expected number of negative cultures calculated from the first term of the Poisson distribution; b″ = expected number of negative cultures calculated from the second term of the Poisson distribution.

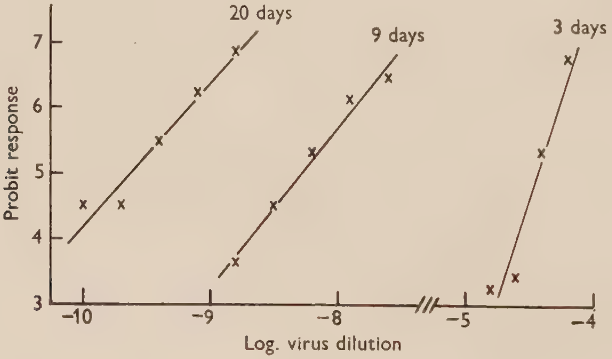

Fig. 1. Log dose-probit response curves of adenovirus titrations read after 3, 9 and 20 days incubation.

are shown in Fig. 1. The 9- and 20-day curves vary in position along the abscissa but both show good agreement with the slope of the one-particle curve. The 3-day curve, on the other hand, differs markedly from these both in position and in slope. The difference between early and late readings is also illustrated by the inverse trend of χ^2 values for goodness of fit with successive terms of the Poisson distribution. Another difference between early and late effects was revealed by the character of the cytopathic changes which were diffuse from start at the early readings whereas at the later readings, initial focal lesions were followed by gradual spread to the surrounding healthy cells.

The possible influence of age of the cultures on the slope of the dose-response curves was investigated by titrating the early cytopathic effect of the same virus preparation in cell cultures incubated for 7 days before addition of the virus inoculum. The result agreed closely with that of the titration done in cell cultures incubated for 1 day before inoculation. The χ^2 test for goodness of fit with the one-particle curve gave a value of 16·76 ($n=3$; P 0·001) and the slope of the log dose-probit response curve (4·80 ± 0·64) was not significantly different from the early reading obtained in the younger cultures.

Effect of ultraviolet irradiation

Two ml. volumes of a 10^{-2} dilution of the virus preparation were placed in 6·5 cm. diameter Petri dishes and exposed for periods of 30, 60 and 90 sec. to ultraviolet light at a distance of 30 cm. from the source of light (Hanovia sun lamp). The original and each of the irradiated preparations were immediately diluted serially in broth saline in 0·5 log steps and titrated in cultures from a single batch of HeLa cells, four cultures being used for each dilution. The cultures were examined after incubation for 3 and 13 days and the 50% end-points based on cytopathic effect were calculated by the method of Reed & Muench (1938). The results shown in Table 2 reveal that the early cytopathic activity was not significantly affected in any of the samples, whereas the late effect was gradually reduced with increasing exposures to irradiation.

Table 2. *End-points of early and late cytopathic effects of adenovirus treated by varying doses of ultraviolet light*

	Time of exposure to ultraviolet light (sec.)			
	0	30	60	90
Early effect (3-day reading)	4·25*	4·25	4·25	4·00
Late effect (13-day reading)	8·25	7·72	7·58	6·76

* Log 50% doses/ml.

COMMENTS

Dose-response curves of virus titrations based on toxic effects have not, to our knowledge, been studied in detail. Rake & Jones (1944), when studying the toxicity of meningo-pneumonitis virus, observed that titration end-points based on toxic activity followed a steeper slope than that of infectivity titrations of the same agent. A steep slope is also suggested by some of the figures reported by Henle & Henle (1946*a*, *b*) in toxicity titrations of influenza viruses in mice. From the data recently published by Khoobyarian & Walker (1957), on titrations of intravenous toxicity of influenza virus in mice, we have found that the dose-response curve in this system is significantly steeper than the one-particle curve with a slope of the log dose-probit response curve of

$3{\cdot}51 \pm 0{\cdot}78$ and a χ^2 for goodness of fit with the one particle curve of 18·25 ($n=4$; $P<0{\cdot}01$). A search of the literature by Meynell & Stocker (1957) revealed only two further examples of virus titrations with slopes significantly greater than 2. The first example (Parker, Bronson & Green, 1941) refers to the titration of a low virulence strain of vaccinia virus which needed large doses to cause skin lesions in rabbits. The possibility of a toxic nature of these lesions should be kept in mind. In the second example (Luria & Dulbecco, 1949) deviation from the one-particle curve was attributed to multiplicity reactivation of ultraviolet irradiated bacteriophages.

The different slopes of early and late adenovirus titration end-points might result from increasing variation in susceptibility of the cultures with age. This explanation can be ruled out by the fact that titrations of the early effect in cultures of different ages do not vary in slope.

The toxic nature of the cytopathic effect of adenoviruses has been suggested by Harford, Hamlin, Parker & van Ravenswaay (1956) on the basis of electronmicroscopic study of HeLa cells infected with large doses of these agents. This suggestion finds support in the observation by Grayston, Johnston, Smith & Loosli (1956), who pointed out the influence of the number of cells present in the cultures on the degree of cytopathic effect caused by adenovirus type 3 after incubation for 2 days. The inverse relation between number of cells and sensitivity to this effect indicates that multiplicity of infection may play a part in this phenomenon. The same is not the case in titrations based on late cytopathic effect (Pereira, unpublished).

The cytopathic effect of adenovirus in HeLa cells is considered as a manifestation of virus infectivity and our results agree with the hypothesis of initiation of infection by single infective units. The relation between infective units and virus particles cannot be determined without direct counts of the latter. The fact that titration end-points read after 9 and 20 days of incubation vary in position but not in slope may be an expression of the probability of occurrence of virus-cell interactions as a function of time. Further study of this aspect of the problem is in progress.

The distinction between toxic and infective activities is rendered difficult in the system under study by the high degree of susceptibility of HeLa cells to virus infectivity. Such a distinction has, however, been clearly demonstrated in the less susceptible cultures of rabbit kidney cells (Kelly & Pereira, to be published).

Further work is required to extend the present findings to other adenovirus types and to determine whether the toxic effect observed is due to synergistic action of virus particles or to some soluble product present in the virus preparations. That this effect is associated with virus activity is evident from results of serum neutralization tests which are based on early cytopathic effects in the majority of techniques in use.

We are indebted to Drs A. W. Gledhill, A. Isaacs, J. Lindenmann and Miss M. V. Mussett for many helpful suggestions and to Mrs P. A. Palmer and Miss P. K. Pearce for valuable technical assistance.

REFERENCES

Beard, J. W., Sharp, D. G. & Eckert, E. A. (1955). Tumor viruses. *Advanc. Virus Res.* **3**, 149.

Bryan, W. R. (1954). The relative precision of dose-response data in the virus and tumor fields as compared with that in certain other fields of biology. *J. nat. Cancer Inst.* **15**, 305.

Bryan, W. R. (1955). Biological studies on the Rous sarcoma virus. I. General introduction. II. Review of sources of experimental variation and of methods for their control. *J. nat. Cancer Inst.* **16**, 285.

Dulbecco, R. (1952). Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. nat. Acad. Sci., Wash.* **38**, 747.

Feemster, R. F. & Wells, W. F. (1933). Experimental and statistical evidence of the particulate nature of the bacteriophage. *J. exp. Med.* **58**, 385.

Gey, G. O. & Gey, M. K. (1936). The maintenance of human normal cells and tumor cells in continuous culture. *Amer. J. Cancer*, **27**, 45.

Grayston, J. T., Johnston, P. B., Smith, M. E. & Loosli, C. G. (1956). An improved technique for the neutralization test with adenoviruses in HeLa cell cultures. *J. infect. Dis.* **99**, 182.

Haldane, J. B. S. (1939). Sampling errors in the determination of bacterial or virus density by the dilution method. *J. Hyg., Camb.* **39**, 289.

Harford, C. G., Hamlin, A., Parker, E. & van Ravenswaay, T. (1956). Electron-microscopy of HeLa cells infected with adenoviruses. *J. exp. Med.* **104**, 443.

Henle, G., Girardi, A. & Henle, W. (1955). A non-transmissible cytopathogenic effect of influenza virus in tissue culture accompanied by formation of non-infectious hemagglutinins. *J. exp. Med.* **101**, 25.

Henle, G. & Henle, W. (1946). Studies on the toxicity of influenza viruses. I. The effect of intracerebral injection of influenza viruses. *J. exp. Med.* **84**, 623.

Henle, W. & Henle, G. (1946). Studies on the toxicity of influenza viruses. II. The effect of intra-abdominal and intravenous injection of influenza viruses. *J. exp. Med.* **84**, 639.

Henle, W. & Henle, G. (1947). The effect of ultraviolet irradiation on various properties of influenza viruses. *J. exp. Med.* **85**, 347.

Isaacs, A. (1957). Particle counts and infectivity titrations for animal viruses. *Advanc. Virus Res.* **4**, 111.

Khoobyarian, N. & Walker, D. L. (1957). Effect of cortisone on mouse resistance to intravenous toxicity of influenza virus. *Proc. Soc. exp. Biol., N.Y.* **94**, 295.

Kjellen, L. (1956). Studies on adenoviruses (APC-RI-ARD) in tissue culture. Correlation between the amount of virus inoculated and the time needed for production of cellular degeneration. *Arch. Virusforsch.* **7**, 110.

Lauffer, M. A. & Price, W. C. (1945). Infection by viruses. *Arch. Biochem.* **8**, 449.

Luria, S. (1939). Sur l'unité lytique du bactériophage. *C.R. Soc. Biol., Paris*, **130**, 904.

Luria, S. (1940). Méthodes statistiques appliquées à l'étude du mode d'action des ultravirus. *Ann. Inst. Pasteur*, **64**, 415.

Luria, S. E. & Dulbecco, R. (1949). Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated bacteriophage particles. *Genetics*, **34**, 93.

Meynell, G. G. & Stocker, B. A. D. (1957). Some hypotheses on the aetiology of fatal infections in partially resistant hosts and their application to mice challenged with *Salmonella paratyphi-B* or *Salmonella typhimurium* by intraperitoneal injection. *J. gen. Microbiol.* **16**, 38.

Parker, R. F., Bronson, L. H. & Green, R. H. (1941). Further studies of the infectious unit of vaccinia. *J. exp. Med.* **74**, 263.

Peto, S. (1953). A dose-response equation for the invasion of microorganisms. *Biometrics*, **9**, 320.

Rake, G. & Jones, H. P. (1944). Studies on lymphogranuloma venereum. II. The association of specific toxins with agents of the lymphogranuloma-psittacosis group. *J. exp. Med.* **79**, 463.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *Amer. J. Hyg.* **27**, 493.

Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H. & Ward, T. G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. exp. Biol., N.Y.* **84**, 570.

Sheffield, F. W. & Smith, W. (1956). The titration of Lansing poliomyelitis virus in HeLa cell cultures. *Brit. J. exp. Path.* **37**, 5.

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Cell-Wall Lytic Enzymes at Sporulation and Spore Germination in *Bacillus* Species

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SUMMARY: When washed sporulating cells of *Bacillus cereus* were incubated in buffer at 37° in the presence of toluene, a partial autolysis occurred resulting in the freeing of mature and immature spores. The autolysate contained lytic enzymes which attacked vegetative cells and cell-wall preparations, releasing hexosamine-containing peptides of characteristic constitution. The most active enzyme preparations were obtained from sporulating cells incubated for 1–2 hr. in buffer at pH 5·0–6·0. Two water-soluble lytic systems, enzyme V and enzyme S with pH optima near 4·5 and 8·0 respectively, were separated from the autolysate. Enzyme S is probably identical with the lytic system present in spores of *B. cereus* and other *Bacillus* species and further observations on this system are described. When non-sporulating cells of *B. cereus* were incubated under similar conditions no obvious lysis or sporulation occurred and no cell-wall lytic activity could be demonstrated. In growing cultures of *Bacillus cereus*, considerable amounts of hexosamine-containing peptides were released into the medium during the period between the appearance of intracellular spores and free spores. It is suggested that enzyme V may be mainly concerned with the release of free spores from sporangia and enzyme S with the lytic processes which accompany spore germination.

Spore release from sporulating cells is a process which can be observed under the microscope and it occurs by dissolution of the vegetative cell. The process might be due to the independent or combined activity of lytic systems present in the cell and the culture medium. Intracellular and extracellular lytic systems derived from *Bacillus* species have been reported by Greenberg & Halvorson (1955), Nomura & Hosoda (1956) and Norris (1957). The significance of the development of these lytic enzymes in growing cultures is not evident from the reports, although the suggestion is made that the autolytic substance described by Greenberg & Halvorson might be responsible for vegetative cell lysis at sporulation (Nomura & Hosoda, 1956). Powell & Strange (1956) found that an intracellular cell-wall lytic system developed during sporulation of *Bacillus cereus* and, in a further investigation (Strange & Dark, 1957), the partial purification and some properties of a lytic enzyme present in extracts of disintegrated resting-spores were described. It was considered that this enzyme released peptide from the spore coat during spore germination but it was not clear whether it or another enzyme dissolved the vegetative cell wall at sporulation.

In this report evidence is presented that sporulating cells of *Bacillus cereus* contain two cell-wall lytic enzymes, 'enzyme V' and 'enzyme S'. A partial separation of these enzymes is described and their properties compared. Further results are given which support the view that spore peptide (Strange &

Powell, 1954) is associated with the coat of the resting spore (Strange & Dark, 1956) and that it is released during germination or disintegration by enzymic activity of enzyme S.

METHODS

Preparation of cell-free solutions of lytic enzymes from sporulating cells and spores of Bacillus cereus

Cultures of a laboratory strain of *Bacillus cereus* were grown at 37° in 1 l. flasks, each containing 200 ml. of potato extract medium (Robinow, 1951) enriched with $\frac{1}{10}$th vol. of casein hydrolysate and yeast extract (Gladstone & Fildes, 1940), on a rotary shaker as previously described (Powell & Strange, 1956). Incubation was continued until intracellular spore formation was at an advanced stage and spore release had begun. The time of incubation varied from 17 to 19 hr. depending on the appearance of the cells in heat-fixed films stained with carbol fuchsin-nigrosin-methylene blue (Powell, 1950) which were examined at intervals. The sporulating cells were harvested by centrifugation at 0° and washed twice with 0·9 % (w/v) saline and once with distilled water. Washed cells (*c.* 0·9–1·0 g. dry weight from 1 l. medium) were suspended in distilled water (20 ml.). A measured volume of suspension was mixed with an equal volume of McIlvaine's buffer and after the addition of toluene (0·025 ml./ml. suspension) the mixture was incubated at 37°. The effect of pH value and time of incubation on the recovery of enzymic activity from autolysates is described in Results. After incubation, the suspension was centrifuged at 6000 ***g*** for 20 min. and the supernatant fluid filtered through sintered glass (porosity 5/3). The filtrate contained cell-wall lytic enzymes and was stored at −10°.

Partially purified cell-wall lytic enzyme was prepared from disintegrated resting spores of *Bacillus cereus* as described by Strange & Dark (1957).

Determination of enzymic activity. To determine the cell-wall lytic activity of enzyme preparations, a substrate of cell walls from non-sporulating cells of *Bacillus cereus* was used. The method for the preparation of cell walls was described in a previous paper and spontaneous lysis was prevented by heating for 15 min. at 100° (Strange & Dark, 1957). Buffer systems in the pH range 3·0–9·6 were: McIlvaine's (0·1 M-citric acid + 0·2 M-Na_2HPO_4), pH 3·0–8·0; 0·1 M-tris-(hydroxymethyl)aminomethane/HCl (Gomori, 1946), pH 8·0–9·0; 0·5 M-Na_2CO_3/$NaHCO_3$, pH 9·6. The effect of the lytic enzymes on the substrate was determined by estimation of the soluble combined amino-sugars released (Strange & Dark, 1957) or by turbidity measurements in a Coleman Junior spectrophotometer at 450 mμ. For turbidity measurements a suspension (2·5 ml.) containing buffer (1 ml.) enzyme solution, cobalt sulphate, (50 μg. Co^{++}) and sufficient substrate to give a $-\log T$ reading of *c.* 0·35 (*c.* 1·5 mg.) was prepared in an optical cuvette (12 × 75 mm.). A control solution containing enzyme but no substrate was prepared to determine turbidity changes caused by precipitation which occurred mostly at acid pH values and usually increased during incubation. A second control tube contained substrate without enzyme. The optical densities of the three tubes were measured and they were incubated at 56°. Readings were taken at intervals after the tubes had been rapidly cooled to 15° and lysis was expressed as percentage decrease of turbidity, allowance being made for changes due to precipitation of the enzyme preparation. The maximum decrease in turbidity varied from 55 to 75 % according to the particular preparation of cell walls. When a number of enzyme solutions were compared they were tested under identical conditions and their activities expressed in terms of the time (min.) taken to produce 50 % of the maximum possible lysis.

Analytical methods. These were as described in a previous paper (Strange & Dark, 1957), but the method for the determination of $\alpha\epsilon$-diaminopimelic acid (DAP) was modified. An acid-hydrolysed sample containing 10–60 μg. DAP was chromatographed on Whatman no. 1 paper using the technique of Rhuland, Work, Denham & Hoare (1955). After reaction with ninhydrin in acetone, the olive-green spot corresponding in position to that of a standard of DAP was cut out and dropped into a test tube. Distilled water (1 ml.), glacial acetic acid (1 ml.) and acid ninhydrin solution (1 ml.; Chinard, 1952) were added and the tube heated at 100° for 5 min. (Work, 1957). After cooling, glacial acetic acid (3 ml.) was added and the yellow solution freed from paper by centrifugation. The optical density of the solution was determined at 430 mμ. against a blank prepared from a non-coloured area of the chromatogram and compared with those of solutions prepared from known amounts of DAP treated in the same way.

RESULTS

The release of soluble components from vegetative cell walls of Bacillus cereus *during sporulation*

Vegetative cell walls of *Bacillus cereus* contained 30–35 % amino sugars (as glucosamine) and 3–4 % DAP. During vegetative growth, small amounts of these substances were present in the culture medium, but during sporulation the amounts increased rapidly. Shaken cultures of *B. cereus* incubated for 10·5, 17·75, 18·75 and 22 hr. at 37° were centrifuged and the supernatant fluids filtered through sintered glass (porosity 5/3). Samples of each filtrate were hydrolysed with 6N-HCl and analysed for hexosamine and DAP (Table 1). After growth for 22 hr. free spores predominated and the concentration of hexosamine and DAP in the culture filtrate was four times that found in the 10 hr. filtrate from vegetative cells. When samples of the culture filtrates were dialysed, it was found that the bulk of these constituents were non-diffusible indicating that they were present in components of relatively high molecular weight. To obtain these components free from other high molecular weight substances present in the culture medium, washed sporulating cells were allowed to partially autolyse in buffer, pH 6·0, containing toluene for 20 hr. at 37°. After removal of the cells by centrifugation and filtration through sintered glass, protein was precipitated by saturation of the solution with ammonium sulphate. The protein-free filtrate was dialysed exhaustively against distilled water at 2° and freeze-dried. A white solid was obtained (97 mg. from

1·74 g. dry wt./sporulating cells) which contained 45 % amino sugars (as glucosamine), 12·7 % carbohydrate excluding hexosamine (as glucose) and 4·2 % DAP. After acid hydrolysis followed by paper chromatography, DAP, glutamic acid, alanine, glucosamine and a substituted hexosamine (Strange, 1956) were found to predominate with small amounts of other amino acids. The composition of this material was very similar to that of the peptides released from the isolated cell walls of *Bacillus cereus* by the enzyme present in spores (Strange & Dark, 1957).

Table 1. *DAP and amino sugar contents of acid-hydrolysis culture medium—filtrates from vegetative and sporulating cells of* Bacillus cereus

Incubation period of culture (hr.)	State of cells	Dry wt. cells/ml. (mg.)	µg./ml. culture medium filtrate	
			DAP	Amino sugars (as glucosamine)
10·5	Vegetative forms	1·37	1·8	20·0
17·75	Intracellular spores	1·40	3·0	24·0
18·75	Intracellular spores	1·40	4·4	32·0
22·0	Free spores	1·30	9·0	85·0

Activity of the cell-free preparations of lytic enzymes from sporulating cells

It was established that the soluble products released during partial autolysis of washed sporulating cells of *Bacillus cereus* included hexosamine-containing peptides and cell-wall lytic enzymes. Preliminary experiments with cell-free preparations indicated that activity was very much greater at 56–58° than at 37° and that cobalt ions were stimulatory (see below). The conditions required to give maximum recovery of lytic activity were defined by investigation of the effect of pH value, time of incubation and the presence of toluene on the autolysis of sporulating cells. A series of suspensions (6 ml.) containing 20 mg. dry wt. of sporulating cells/ml. in buffer pH 5·4–7·5 with toluene were incubated in parallel with similar suspensions without toluene. Samples (1 ml.) were removed at intervals, freed from cells and tested for lytic activity by turbidity measurements at pH values of pH 4·5 and 7·0. The results expressed in terms of the time (min.) taken by 0·1 ml. autolysate to produce 50 % maximum lysis (Table 2), indicated that optimum conditions for recovery of lytic enzymes were present when a suspension of sporulating cells buffered at pH 5·0–6·0 was incubated for 1–4 hr. at 37°. Lytic activity decreased during prolonged incubation but this decrease was lowest at pH values near 5·0. At acid pH values toluene had little effect, but above pH 6·0 its presence substantially increased the yield of lytic activity. Examination of films stained for spores (Powell, 1950) and cell walls (Webb, 1954) indicated that after incubation for 1 hr. at 37°, complete or substantial spore release occurred under all the conditions used and that vegetative cell walls were dissolved. Of the free spores, a variable number did not stain red in outline which is typical of resting spores but appeared as weakly staining blue rings. It is possible that these were immature spores released from the sporangia because of the relatively high concentration of lytic enzymes present in the thick suspensions, before the spore coats had properly developed. They appeared to be as refractile as normal spores when examined in wet preparations. Germinated spores, i.e. forms permeable to stains (Powell, 1950) were most abundant in autolysates incubated in the presence of toluene and vegetative cell structure persisted at pH values of 6·9 and 7·5 in the absence of toluene. Investigation of autolysis at pH values of 3·0 and 4·5 were also made. At pH 3·0 little lysis occurred and only a small amount of lytic enzyme was recovered. Substantial autolysis occurred at pH 4·5 but the activity recovered was less than at pH 5–6.

Table 2. *Concentration of lysins in cell-free preparations from partial autolysates of sporulating cells of* Bacillus cereus

Cell-free autolysates (0·1 ml. derived from 2 mg. dry wt. sporulating cells) tested for lytic activity. Lytic test suspensions (2·5 ml.) contained McIlvaine's buffer, pH 4·5 or 7·0 (1 ml.), *B. cereus* cell walls (1·5 mg.) Co^{++} (50 μg.). Incubation at 56°.

		Time (min.) to produce 50 % max. lysis by 0·1 ml. autolysate					
		Lytic tests at pH 4·5			Lytic tests at pH 7·0		
pH during autolysis	Toluene	1*	4	20	1*	4	20
5·4	+	22·5	26·5	31·5	37·5	66·0	132
5·4	–	17·0	26·5	39·0	22·5	57·0	375
6·1	+	24·0	26·5	375	34·5	79·5	375
6·1	–	19·5	22·5	†	27·0	64·5	375
6·9	+	25·5	84·0	†	45·0	124·5	†
6·9	–	48·0	†	†	†	†	†
7·5	+	180	375	†	63·0	375	†
7·5	–	375	375	†	†	†	†

* Incubation time (hr.) of autolysate at 37°.
† No detectable activity.

The rate of cell-wall lysis in the presence of 0·025–0·1 ml. of a typical cell-free autolysate (pH 6·0, 1 hr.) as determined by turbidity measurements is shown in Fig. 1. In the presence of 0·1 ml. of the preparation (derived from 2·6 mg. dry wt. of sporulating cells), the optical density of a suspension of cell walls (1·46 mg./2·5 ml.) buffered at pH 4·6, was decreased from 0·365 to 0·154 (58 % decrease) in 1 hr. at 56°. Films prepared after incubation and stained for cell walls (Webb, 1954) showed only amorphous debris. The optical density of a control suspension incubated without enzyme remained stationary. The effect of increasing concentrations of Co^{++} (0–200 μg./ml.) on lytic activity was determined at pH values of 5·0 and 7·0. In both cases stimulation occurred which reached a maximum at a concentration of 100 μg. Co^{++}/ml. (Fig. 2). When acetate buffer (0·5M) replaced McIlvaine's buffer at pH 5·0, a similar stimulation occurred in the presence of Co^{++} which showed that citric acid was not concerned with this effect.

Some inhibition of activity occurred when the salt concentration of the system was increased. In McIlvaine's buffer (pH 4·5) containing KCl in concentrations of 0·05, 0·1, 0·2 and 0·4 M, the time taken to produce 50% maximum lysis of 1·3 mg. cell walls by 0·1 ml. autolysate was 13·5, 16·5, 20 and 24 min. respectively, compared with 13·0 min. in the absence of KCl. The effect of the added salt on the pH value of the system was not sufficient to account for the inhibition. Addition of NaCl or increasing the concentration of the buffer salts also reduced activity. Enzymic activity was completely destroyed by heating preparations at 100° for 15 min.

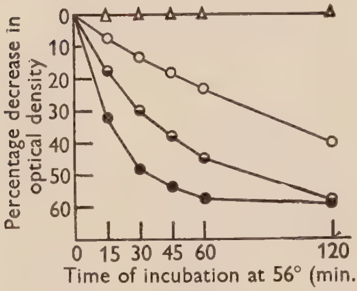


Fig. 1

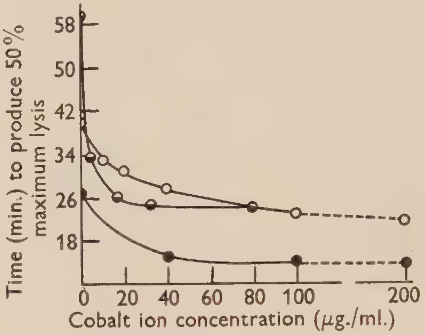


Fig. 2

Fig. 1. The rate of lysis at 56° of *Bacillus cereus* cell walls in the presence of various amounts of cell-free autolysate from *B. cereus* sporulating cells: the decrease in turbidity of a cell-wall suspension (1·46 mg./2·5 ml.) buffered at pH 4·6 and incubated with 0·025 ml. (○), 0·5 ml. (◒), 0·1 ml. (●) of the preparation; cell-wall control, △.

Fig. 2. Effect of increasing amounts of cobalt ions (0–200 µg./ml.) on the activity of lytic enzymes from *Bacillus cereus* sporulating cells: results expressed in terms of the time taken to produce 50% maximum lysis of cell walls (1·46 mg./2·5 ml.) by a cell-free autolysate (0·1 ml.) in McIlvaine's buffer, pH 5·1 (○), in acetate buffer, pH 5·0 (●), in McIlvaine's buffer, pH 7·0 (◒). Incubation at 56°.

Effect of pH *value on the activity of lytic enzymes from sporulating cells*

The effect of pH value on lytic activity was determined turbidimetrically and also by estimation of the combined hesoxamine released from the cell-wall substrate. The enzyme preparations contained combined hexosamine and this interfered with subsequent analyses; active material was freed from it by precipitation with $(NH_4)_2SO_4$ (1·0 saturation) at 0°. The precipitate was separated by filtration through sintered glass (porosity 5/3) and dissolved in 0·01 M-$NaHCO_3$. When this solution was diluted to the same volume, it was as active as the original preparation but contained an insignificant amount of hexosamine. A series of suspensions (2·5 ml.) buffered in the pH range 3·0–9·6, containing substrate (3·65 mg.), enzyme (0·05 ml. from 1·3 mg. sporulating cells) and Co^{++} (50 µg.) were prepared and optical density readings taken. After incubation at 56° for 2 hr. in parallel with controls, further optical density readings were taken, pH determinations were made and the suspensions were centrifuged. A measured volume (0·2 ml.) of each supernatant fluid was dried at 100°, hydrolysed with 6 N-HCl for 2 hr. at 100° and analysed for hexosamine. Decrease in turbidity and release of hexosamine followed a similar course and were greatest at pH values near 4·5 and 8·0 (Fig. 3). It thus appeared that two distinct lytic systems were released from sporulating cells and attempts were made to separate them.

Partial separation and properties of two lytic systems from sporulating cells

Attempts to separate the two lytic systems by fractionational precipitation with $(NH_4)_2SO_4$ at 0° were not very successful. A separation based on the relative solubilities of the enzymes at pH 3·0 was more satisfactory and was

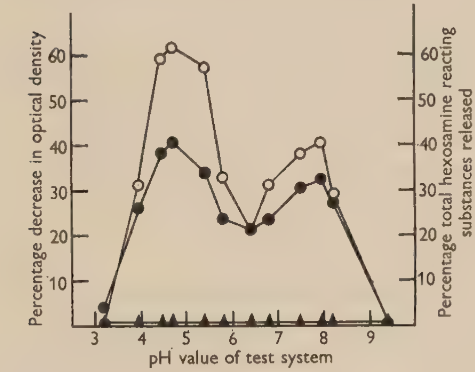


Fig. 3

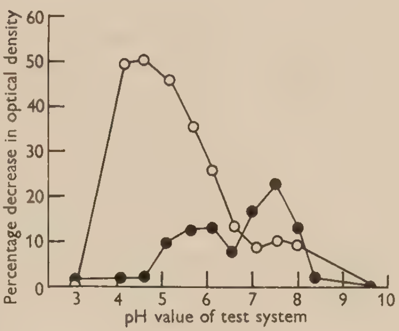


Fig. 4

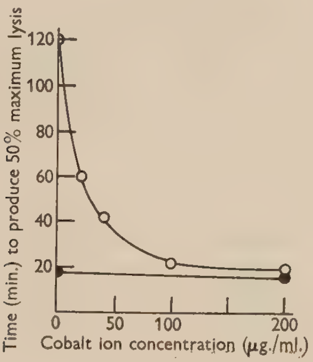


Fig. 5

Fig. 3. Effect of pH on the activity of lytic enzymes from *Bacillus cereus* sporulating cells in terms of the percentage total combined hexosamine released (○) and percentage decrease in turbidity (●) during incubation for 2 hr. at 56° with cell walls (3·65 mg./2·5 ml.), cell wall control (▲).

Fig. 4. Effect of pH value on the activity of two lytic enzymes separated from a 1 hr. autolysate (pH 6·0) of *Bacillus cereus* sporulating cells: percentage reduction in turbidity of cell-wall suspensions (1·4 mg./2·5 ml.) with 0·1 ml. (from 2·5 mg. cells) of enzyme V in 1 hr. at 56° (○); with 0·1 ml. (from 5 mg. cells) of enzyme S in 2 hr. at 56° (●).

Fig. 5. Effect of increasing amounts of cobalt ions (0–200 µg./ml.) on the activity of two lytic enzymes from *Bacillus cereus* sporulating cells: time taken to produce 50% maximum lysis of cell walls (1·5 mg./2·5 ml.) by 0·1 ml. (from 2·5 mg. cells) of enzyme V at pH 4·5 (●); by 0·1 ml. (from 5·0 mg. cells) of enzyme S at pH 6·2 (○). Incubation at 56°.

achieved as follows: cooled cell-free autolysate (1 vol.) was added to cold McIlvaine's buffer (2 vol.) pH 3·0, and the mixture allowed to stand for 30 min. at 0°. The precipitate which formed was separated by centrifugation and dissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). This fraction was reprecipitated from the solution by the addition of an equal volume of buffer (pH 3·0), and redissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). The supernatant fluid from the first precipitate at pH 3·0 was saturated with solid $(NH_4)_2SO_4$ and allowed to stand for 1 hr. at 0°. The precipitate which formed was separated by filtration through sintered glass, washed with 0·9 saturated $(NH_4)_2SO_4$ solution and dissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). The effect of pH value on the activity of these two fractions, i.e. the pH 3·0 insoluble material and the $(NH_4)_2SO_4$ precipitated pH 3·0 soluble material, was determined turbidimetrically. It was found that maximum lysis occurred at pH values near 8·0 and 4·5 respectively (Fig. 4). The two enzymes are subsequently referred to as 'enzyme S' (pH 3·0 insoluble) and 'enzyme V' (pH 3·0 soluble). When the separation procedure was applied to a number of cell-free autolysates it was found that the relative amounts of the two enzymes varied considerably, depending on the time of incubation and pH value during autolysis. Another factor which could not be assessed but which probably affected the amounts of lytic enzyme recovered was the precise state of the sporulating cells before autolysis, e.g. the proportion of mature to immature intracellular spores. Enzyme V appeared to predominate in preparations from autolysates in buffer pH 5·0–6·0 incubated for 1–2 hr. and usually only small amounts of enzyme S were separated. When the suspensions of sporulating cells were incubated for longer periods, the ratio of enzyme S to enzyme V was greater. The two enzymes could also be distinguished by the effect of Co^{++} on their activities. Enzyme S was progressively more active in the presence of increasing amounts of Co^{++} (0–200 μg./ml.), whereas the activity of enzyme V was unaffected (Fig. 5).

Effect of lytic enzymes from sporulating cells on intact vegetative cells

Cell-free autolysates from sporulating cells attacked suspensions of washed vegetative cells of *Bacillus cereus*. The decrease in turbidity during incubation at 58°, however, was small: values of *c.* 16% decrease were obtained after 1 hr. and no further decrease occurred when incubation was continued for several hours. It appeared likely that at 58° the vegetative cell contents were coagulated and that the decrease in turbidity represented only the dissolution of cell walls. Examination of stained films of the suspensions after incubation with enzyme showed that the walls were dissolved, leaving the cell contents as Gram-negative fragments. A similar result was obtained when cell suspensions were heated at 100° for 15 min. before incubation at 56° with lytic enzyme. At 37° in the presence of thiomersalate (1/5000) the lytic enzymes had no visible effect for several hours, but after 16 hr. a 60% decrease in turbidity occurred, whereas the turbidity of a control suspension incubated for the same period without enzyme increased by 10%. Stained films of the suspensions incubated with lysins at 37° showed no evidence of residual cell structures. The lytic enzymes had no obvious effect on suspensions of viable or heat-killed vegetative cells of *B. megaterium* at either 37 or 56°.

Investigation of non-sporulating cells and culture medium filtrate for the presence of lytic enzymes

Washed *Bacillus cereus* harvested from potato-extract medium after growth for 9·5 hr. at 37° appeared as Gram-positive rods in stained films and showed no evidence of sporulation. After incubation of a suspension in buffer (pH 6·0) containing toluene for 4 hr. at 37° the organisms showed no evidence of lysis but were now Gram-negative. Examination of films stained by Webb's (1954) method showed that the cell walls had not been dissolved. A cell-free preparation from the incubated suspension had no detectable lytic activity against *B. cereus* cell walls. The culture medium filtrate from sporulating cells of *B. cereus* after growth for 18 hr. was saturated with ammonium sulphate and the resulting precipitate was tested for lytic activity. An insignificant amount of lytic enzyme was present.

Comparison of the lysins from sporulating cells with the lysin present in spores

It was previously found that a cell-wall lytic enzyme present in extracts of disintegrated resting spores of *Bacillus cereus* and other *Bacillus* spp. showed optimum activity at pH 7–8 in the presence of cobalt ions at 58° (Strange & Dark, 1957). Re-examination of the effects of pH value and increasing amounts of Co^{++} on the activity of this lysin indicated that its properties were very similar to those of enzyme S (Figs. 6, 7). Both the spore enzyme and enzyme S were insoluble at pH 3·0 and this property had been used for the

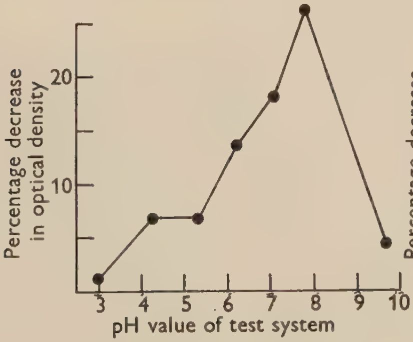


Fig. 6

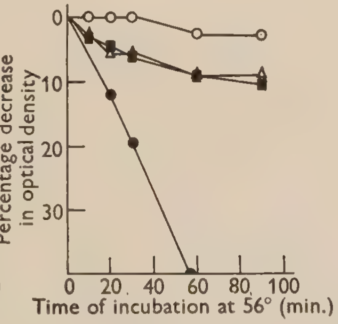


Fig. 7

Fig. 6. Effect of pH value on the activity of lytic enzyme in extracts of disintegrated resting spores of *Bacillus cereus*: percentage decrease in turbidity of cell-wall suspensions (1·3 mg./2·5 ml.) containing buffer (1 ml.) cobalt ions (20 μg.) and enzyme from 5 mg. spores, after incubation for 2 hr. at 56°.

Fig. 7. Effect of cobalt on the activity of lytic enzyme from resting spores of *Bacillus cereus*: percentage decrease in turbidity of cell-wall suspensions (1·3 mg./2·5 ml.) containing enzyme from 2·5 mg. spores at pH 4·9 without cobalt (○) and with 20 μg. Co^{++}/ml. (△); at pH 7·1 without cobalt (■) and with 20 μg. Co^{++}/ml. (●).

separation of spore enzyme from spore peptide. The possibility that some enzyme V was also present in resting spores was checked by testing the activity of material precipitated from spore extracts with $(NH_4)_2SO_4$ (1·0 saturation). The results showed that resting spores contained little or no enzyme V.

Evidence that peptide is released from spore coats by enzymic activity during mechanical disintegration of spores

In previous reports (Powell & Strange, 1956; Strange & Dark, 1957) it was suggested that the function of enzyme S in spores is to release peptide from the spore coat during germination and that the presence of soluble peptide in aqueous extracts of disintegrated spores is due to the attack of enzyme S on the spore coat during mechanical disintegration. However, Powell & Strange (1957) were unable to demonstrate a cell-wall lytic enzyme similar to enzyme S in spores of *Bacillus sphaericus*. Spore extracts did not attack vegetative cell walls of this organism or those of *B. cereus*. Spore peptide was found in extracts from *B. sphaericus* spores, while the coats contained relatively small amounts of peptide as indicated by their low content of DAP and amino sugars. It seemed possible, though unlikely, that in *B. sphaericus* the spore peptide was not attached to the spore coat and appeared in spore extracts independently of a lytic system. This possibility can now be excluded on the following grounds: enzyme S in spores of *B. cereus* was inactive at pH 9·6 (see above). If in fact the detachment of the peptide from the spore coat during disintegration depends on the activity of enzyme S, disintegration at pH 9·6 should yield spore coat preparations of relatively high DAP and hexosamine content. This was found to be the case. Thus spore suspensions of *B. cereus* and *B. sphaericus* (NCTC 9602) in distilled water (*c.* 10 mg. dry wt./ml.) were disintegrated in a Mickle (1948) tissue disintegrator and the spore coat fractions isolated by centrifugation. After washing several times in saline (0·9 % NaCl, w/v) and distilled water, the coats were freeze-dried. Similar preparations were made from spores suspended in 0·5 M-$Na_2CO_3/NaHCO_3$ (pH 9·6) and also, in the case of *B. cereus*, from a suspension in 0·5 M-sodium acetate/acetic acid (pH 5·0). During the washing of the coats from buffered homogenates, possible enzymic activity was minimized by using dilute solutions (1/10) of the appropriate buffers and distilled water as the final wash. Analyses (Table 3)

Table 3. *Hexosamine content of acid-hydrolysed spore coats of* Bacillus cereus *and* B. sphaericus *NCTC* 9602 *isolated from spores disintegrated at different* pH *values*

Source of coats	pH during disintegration	Hexosamine (g./100 g. spore coats)
Bacillus cereus spores	5·0	3·0
B. cereus spores	7·4 (water)	2·3
B. cereus spores	9·6	6·2
B. sphaericus (NCTC 9602) spores	7·4 (water)	2·3
B. sphaericus (NCTC 9602) spores	9·6	6·5

of the acid-hydrolysed coats showed that considerably more hexosamine was present in those from spores disintegrated at pH 9·6 than at pH 7·4 or pH 5·0. When coats from the pH 9·6 homogenates were suspended in buffer (pH 7·1) containing Co^{++} (10 μg./ml.) and incubated for 2 hr. at 56° a considerable release of hexosamine-containing components occurred (Table 4). However, when the coats were heated at 100° for 15 min. before incubation in buffer, a much smaller release of material occurred. Addition of the homologous spore-extract or lysin S to heat-inactivated spore coats caused release of hexosamine-containing material in similar amounts to those released spontaneously from non-heated coats (Table 4).

Table 4. *Release of combined hexosamine from spore coats of* Bacillus cereus *and* B. sphaericus *NCTC* 9602

Suspensions (0·5 ml.) contained McIlvaine's buffer (pH 7·1), 0·2 ml., cobalt, 5 μg. and spore coats. Incubation, 2 hr. at 58°. Hexosamine was determined in the supernatant fluids after hydrolysis with 6N-HCl for 2 hr. at 100°. The *Bacillus cereus* spore extract was freed from hexosamine, whereas that from *B. sphaericus* was not: this is allowed for in the results.

Source of spore coats	Wt. (mg.)	Source of extract	Substrate hexosamine (μg.)	Hexosamine released (μg.)	Percentage substrate hexosamine released
Bacillus cereus	5	—	310	108	35
B. cereus	5 (H)	—	310	12	4
B. cereus	5 (H)	6 mg. *B. cereus* spores	310	79	26
B. sphaericus	5	—	325	225	69
B. sphaericus	4·9 (H)	—	304	62	20
B. sphaericus	4·9 (H)	5 mg. *B. sphaericus* spores	304	290–56*	77

(H) = heated at 100° for 15 min.

* 56 μg. of hexosamine present in the added spore extract.

DISCUSSION

The results of the present work establish that there are two distinct lytic systems present in sporulating cells of *Bacillus cereus*. Neither of these was detected in non-sporulating vegetative cells and only small amounts were found in concentrates of the filtered culture medium from intact, i.e. non-autolysed sporulating cells. Lysis of the sporangia to allow spore release appears to be due to one or both of these intracellular lytic enzymes. One of these enzymes (S) is probably identical with that found in extracts of disintegrated *B. cereus* resting spores and might have been derived from immature spores which lysed under the experimental conditions used. The mode of attack of both enzyme S and enzyme V is readily distinguishable from that of the lytic principle associated with non-sporulating cells of a strain of *B. cereus* (Norris, 1957) which dissolved the protoplasts of vegetative cells of a number of *Bacillus* spp., leaving the cell walls. Dr J. R. Norris (private communication) has found that these residual cell walls are dissolved by a preparation of *B. cereus* enzyme S which we supplied. It is not possible to compare the activity of either enzyme S or V with that of the lytic substance from *B. cereus* var. *terminalis* described by Greenberg & Halvorson (1955), since these authors did not define the lytic substrate. The lytic enzyme demonstrated in autolysates of *B. subtilis* vegetative cells (Nomura & Hosoda, 1956) resembled enzymes S and V in its mode of action, attacking vegetative cell-wall preparations of this organism; this system appeared in non-sporulating cultures. We have found that buffered suspensions of washed vegetative cells of a laboratory strain of *B. subtilis* showing no evidence of sporulation, autolysed on incubation at 37° or 56°, whereas suspensions of vegetative cells of *B. megaterium* and *B. cereus* were stable. Suspensions of cell walls isolated from vegetative *B. subtilis* also lysed spontaneously and this was not completely prevented by heating them at 100° for 15 min. (Strange & Dark, 1957).

The action of enzymes S and V on heat-killed vegetative cells and cell walls resembles that of lysozyme. Salton (1953) showed that the walls of heat-killed *Bacillus megaterium* were dissolved by lysozyme but the coagulated cell contents were left unchanged. However, whereas lysozyme was much more active against intact cells of *B. megaterium* and *B. subtilis* than those of *B. cereus* (Salton, 1956), enzymes S and V attacked intact cells of *B. cereus* but not those of *B. megaterium*. Enzyme S from *B. cereus* spores attacked the isolated cell walls from all three organisms (Strange & Dark, 1957).

The results of previous work with *Bacillus sphaericus* (Powell & Strange, 1957) showed that this organism differed biochemically in a number of ways from other members of the group. For example, the peptide component present in vegetative cell walls contained lysine instead of α,ϵ-diaminopimelic acid. *B. sphaericus* spore peptide did contain α,ϵ-diaminopimelic acid and resembled peptides present in vegetative cell walls and spores of other *Bacillus* spp. It has now been shown that *B. sphaericus* spores contain a lytic enzyme with similar properties to those of enzyme S. The inability of this enzyme to dissolve *B. sphaericus* vegetative cell walls might be due to the different composition of the peptide present.

The interpretation of lysis caused by substances of bacterial origin must take into account the possibility that the agent is a bacteriophage or bacteriocin. The enzymes described in the present work attacked substrates of heat-killed bacteria and their optimum temperature for lytic activity was near 56°; they appear to play a definite part in sporulation and spore germination. These properties are akin to those of an enzyme rather than a bacteriophage or bacteriocin.

We wish to thank Mrs Joan F. Powell for reading and criticizing the manuscript. We are grateful for her interest in this work.

REFERENCES

Chinard, F. P. (1952). Photometric estimation of proline and ornithine. *J. biol. Chem.* **199**, 91.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Gomori, G. (1946). Buffers in the range of pH 6·5 to 9·6. *Proc. Soc. exp. Biol., N.Y.* **62**, 33.

Greenberg, R. A. & Halvorson, H. D. (1955). Studies on an autolytic substance produced by an aerobic spore forming bacterium. *J. Bact.* **69**, 45.

Mickle, H. (1948). Tissue disintegrator. *J. R. micr. Soc.* **68**, 10.

Nomura, M. & Hosoda, J. (1956). Nature of the primary action of the autolysin of *Bacillus subtilis*. *J. Bact.* **72**, 573.

Norris, J. R. (1957). A bacteriolytic principle associated with cultures of *Bacillus cereus*. *J. gen. Microbiol.* **16**, 1.

Powell, J. F. (1950). Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. *J. gen. Microbiol.* **4**, 330.

Powell, J. F. & Strange, R. E. (1956). Biochemical changes occurring during sporulation in *Bacillus* species. *Biochem. J.* **63**, 661.

Powell, J. F. & Strange, R. E. (1957). α, ϵ-Diaminopimelic acid metabolism and sporulation in *Bacillus* species. *Biochem. J.* **65**, 700.

Rhuland, L. E., Work, E., Denham, R. F. & Hoare, D. S. (1955). The behaviour of the isomers of α, ϵ-diaminopimelic acid on paper chromatograms. *J. Amer. chem. Soc.* **77**, 4844.

Robinow, C. F. (1951). Observations on the structure of *Bacillus* spores. *J. gen. Microbiol.* **5**, 439.

Salton, M. R. J. (1953). Cell structure and the enzymic lysis of bacteria. *J. gen. Microbiol.* **9**, 512.

Salton, M. R. J. (1956). Bacterial cell walls. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 81.

Strange, R. E. (1956). The structure of an amino sugar present in certain spores and bacterial cell walls. *Biochem. J.* **64**, 23*P*.

Strange, R. E. & Dark, F. A. (1956). The composition of the spore coats of *Bacillus megaterium, B. subtilis* and *B. cereus*. *Biochem. J.* **62**, 459.

Strange, R. E. & Dark, F. A. (1957). A cell-wall lytic enzyme associated with spores of *Bacillus* species. *J. gen. Microbiol.* **16**, 236.

Strange, R. E. & Powell, J. F. (1954). Hexosamine-containing peptides in spores of *Bacillus subtilis, B. megaterium* and *B. cereus*. *Biochem. J.* **58**, 80.

Webb, R. B. (1954). A useful bacterial cell wall stain. *J. Bact.* **67**, 252.

Work, E. (1957). Reaction of α, ϵ-diaminopimelic acid and its homologues with ninhydrin in acid solution. *Biochem. J.* (in the Press).

(*Received* 25 *April* 1957)

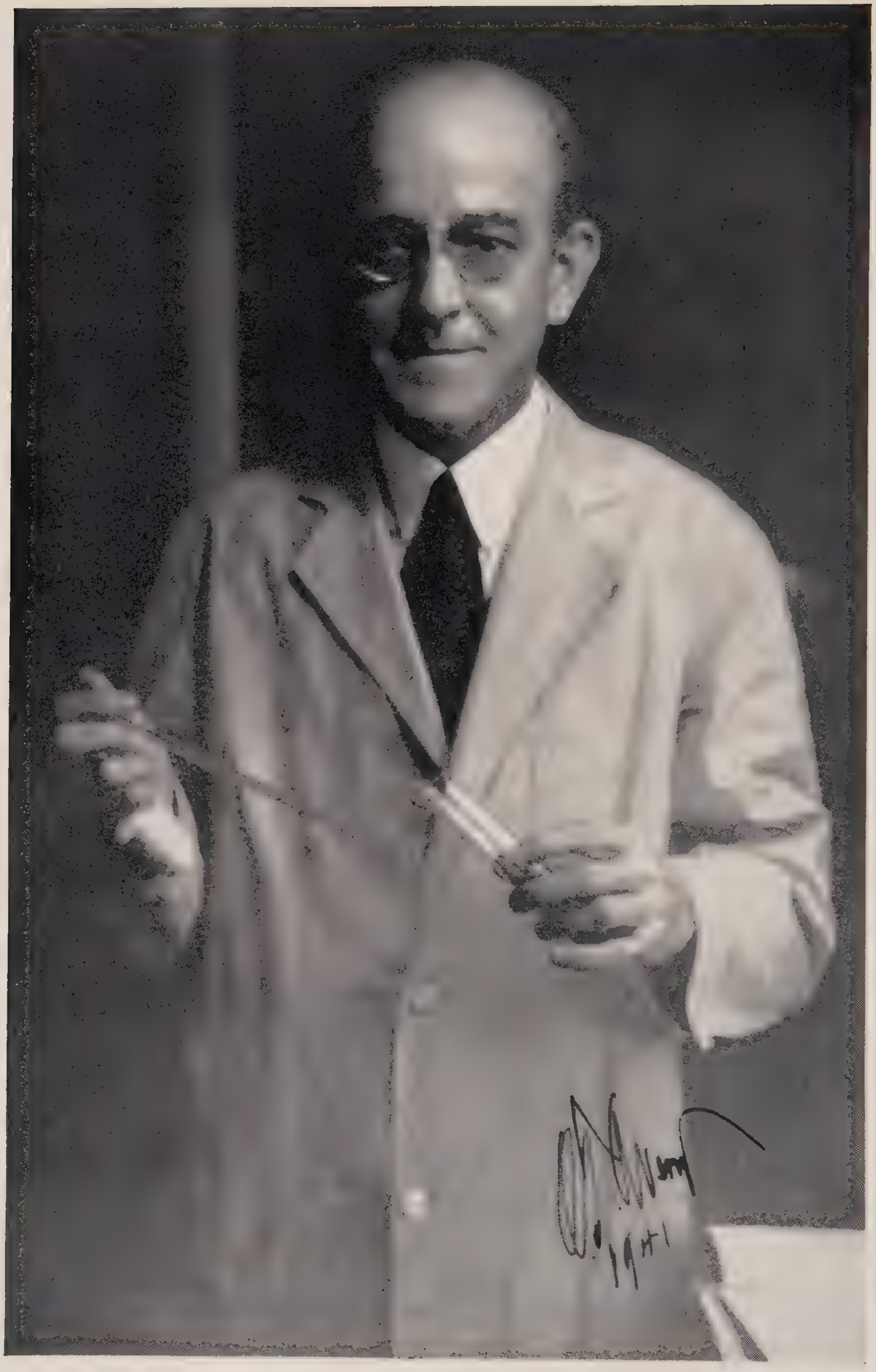
CORRIGENDUM

BAILEY, L. (1957). *J. gen. Microbiol.* **17**, 39–48

On page 46, lines 29 and 30:

For: ‘the molar ratio of Na:K was 0·81,’

Read: ‘the molar ratio of Na:K ranged from 173–2120,’



OSWALD THEODORE AVERY

(1877–1955)

(*Facing p.* 539)

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OBITUARY NOTICE (1957). *J. gen. Microbiol.* 17, 539–549

Obituary Notice

OSWALD THEODORE AVERY, 1877–1955

Oswald Theodore Avery was born in Halifax, Nova Scotia, on 21 October 1877, and lived there until the age of 10, when his father, who was a clergyman, moved to New York City. Except for a period of 4 years at Colgate University in Hamilton, New York, from which he obtained the A.B. degree in 1900, Avery's life was spent in New York City until the age of 70 when he retired from active investigation to Nashville, Tennessee. He died there on 20 February, 1955.

Avery received the M.D. degree from the College of Physicians and Surgeons of Columbia University in 1904. Following a brief time spent as an assistant in a busy clinical practice, he began his career in bacteriology. He was first employed in a milk control laboratory and at the same time started investigations of respiratory infections as time could be spared from other duties. A year later, in 1906, he accepted a position at the Hoagland Laboratory in Brooklyn, where he began his life-long association with the director, Dr Benjamin White. The Hoagland had the distinction of being the first privately endowed laboratory for bacteriological research in the United States. Avery always spoke with great affection of the time he spent at the Hoagland and of the unique opportunities it was able to provide for him. During his 6 years at the Hoagland Laboratory he published nine papers, mostly in collaboration with White, on a variety of topics including the demonstration of *Treponema pallidum* in syphilitic lesions; *Lactobacillus bulgaricus*, then in vogue as 'the bacillus of long life'; and the immunological reactions of certain vegetable proteins. White developed tuberculosis and was confined to Trudeau Sanatorium where Avery visited him whenever possible, even spending vacations there. An interest in tuberculosis was aroused thereby, and between 1910 and 1913 he and White published three papers on the subject, one of which, concerned with a toxic derivative prepared from tubercle bacillus by extraction in alkaline ethanol, is the first that indicated a pattern he was to follow afterwards with such success, namely, the relationship of chemical composition of bacteria to their biological properties.

Avery's studies on pneumococcus began after he joined the staff of the Hospital of the Rockefeller Institute in 1913. Rufus Cole, the first director and guiding spirit of the Rockefeller Hospital, was dedicated to the ideal of joining fundamental laboratory investigation and the study of diseases of man. He invited Avery to join the staff of the pneumonia service as bacteriologist. Thereafter most of Avery's investigations dealt with the biology of a single bacterial species, pneumococcus, and its relation to disease.

He was not concerned directly with day to day clinical problems although keenly aware of the trials of the resident staff in the care of acutely ill patients.

He rejoiced with them at the spectacular successes that occurred with increasing frequency as specific serum therapy was improved and extended to new pneumococcal types. He also shared their feelings of despair when therapeutic resources failed, as so frequently happened before the advent of sulphonamide compounds and their chemotherapeutic followers.

The elaboration, structure and biological significance of the capsular polysaccharides of pneumococci were the dominant themes of Avery's scientific career from the time he went to the Rockefeller Hospital at the age of 35 until he died 43 years later. In collaboration with a modest number of associates over this long period he published many papers on aspects of pneumococcus that were not related directly to the polysaccharides, but he never varied in his main interest and always returned to it. Except for the period of 1935–40 when his work was seriously interfered with because of Graves's disease, there were only 5 years between 1915 and 1946 when one or more papers dealing with pneumococcal polysaccharides did not appear. In collaboration with A. R. Dochez, he studied the relation of the capsular material, then chemically unidentified, to type differentiation and specific immunity. Their first paper on the specific soluble substances (SSS), later identified as complex carbohydrate polymers, was published in 1917. In 1922 there began a most fruitful collaboration, at first with M. Heidelberger and two years later with W. F. Goebel, on the relationship of chemical constitution to immunological specificity of polysaccharides. This body of work, classic in both concept and execution, is recognized along with the contributions of Landsteiner as forming the main pillars of modern immunochemical theory.

It should be recalled that prior to the observations of Dochez, Avery and Heidelberger on the specific soluble substances of pneumococcus, proteins were generally accepted as the determinants of immunological specificity. Avery commented frequently on the scepticism that greeted the description of the antigenic specificity of polysaccharides and likened it to the doubt that was generally expressed as to the protein nature of enzymes following Sumner's report of the crystallization of bean urease and the crystallization of trypsin by Northrop. He anticipated that even greater scepticism would be manifested on the announcement of the genetic specificity of deoxyribonucleic acids. The memory of the turmoil that attended the description of the immunological specificity of polysaccharides and the protein nature of enzymes haunted him for several years before and after the publication in 1944 of the identification of the principle responsible for type transformation in pneumococci with deoxyribonucleic acids.

Avery's approach to a problem was characterized by a prolonged period of thought and discussion, but the strictest economy of mechanical effort. He was scornful of ill-planned experiments that commonly consisted as he would say, in 'taking something out of one tube and putting it into another'. The approach was thoroughly mulled over with his associates, the goal clearly defined and finally a 'protocol' experiment set up, which, if it failed to yield the expected results, pointed unequivocally to another and often more interesting problem. There would then ensue extensive discussion of the implications of the observation, and it was here that another aspect of Avery's genius became apparent. In contrast to the cautious, economical approach to a laboratory experiment, his imagination was now fired by a fact. He would explore the theoretical implications exhaustively in animated discussion, though usually ending up with the homely reminder that the blowing of bubbles is all right as long as one remembers to prick them oneself.

The meticulousness that Avery brought to experimental design applied also to the writing of scientific papers and to his rare public addresses. Indeed, his concern at such times amounted almost to neurosis. The final products are models of clarity in organization and expression and perhaps do not reflect the repeated, careful balancing of one word or sentence against another, and the discarding of drafts until the final product pleased his critical mind and aesthetic sense. His disenchantment with the 'raking over of old bones', when a paper was at last ready for the publisher, was often dismissed with a wry smile and a remark to the effect that if there were really something to say it would be easy to say it.

Underlying Avery's studies on the chemical and immunological properties of the capsular polysaccharides was an even more fundamental interest in their dominant role in the virulence of pneumococcus. Studies from his laboratory had demonstrated that the antiphagocytic properties of the capsule were chiefly responsible for the ability of pneumococcus to survive and grow in the non-immune host. Means were sought, therefore, to neutralize or destroy the antiphagocytic activity—to make a specific approach to the prevention and therapy of the disease in man. There was deep disillusionment on the outlook for antibacterial chemotherapy with the rejection of ethylhydrocupreine (optochin) because of its frequent and severe toxicity for the optic nerve and the rapidity with which pneumococcus acquired resistance to it. At the time other approaches to chemotherapy seemed barren because the many additional compounds that had been tested showed toxic indices that were even more unfavourable. The preparation, refinement and effective use of type-specific antipneumococcal serum, therefore, occupied much of the early effort of the laboratory and its associated clinical service. Avery's contributions in this effort were crucial. The principles were clearly laid down in a monograph on the prevention and serum treatment of acute lobar pneumonia published in 1917 in collaboration with Chickering, Cole and Dochez.

During the succeeding 20 years marked improvements were made in serum therapy for infections caused by many pneumococcal types. Serum for type III pneumonia, one of the commonest and most lethal varieties, however, had little or no therapeutic effect, because the capsular polysaccharide is produced in very large amounts by type III organisms and is weakly antigenic in animals used for the production of antiserum. In collaboration with R. J. Dubos, Avery devised what to this day remains a unique approach to rational chemotherapy. They announced in 1930 that the capsular polysaccharide of type III pneumococcus is hydrolysed by a specific enzyme produced adaptively by a soil bacillus when grown in the presence of type III polysaccharide as energy source. The following year they reported that the type III enzyme possesses a remarkable protective effect against type III pneumococcal infections in mice. Subsequently, rabbits and monkeys were shown to be protected or cured. Application of this brilliant concept to the treatment of human disease was not made, because discovery of the therapeutic effectiveness of sulphonamides occurred while the preparation of the type III enzyme was still in a developmental stage.

Although Avery was concerned chiefly with the capsular polysaccharides and the immunological responses of the host to them, he maintained an active interest in more general reactions of the body to infection and toxic damage, or as he termed it, the chemistry of the host. With Tillett and Goebel in 1930 he reported on the properties of the C or somatic carbohydrate common to all pneumococci irrespective of type, and in the same year Tillett and Francis described a precipitation reaction between the newly discovered C carbohydrate and the serum of patients acutely ill with pneumococcal pneumonia as well as certain other acute infections of unrelated aetiology. This finding indicated that the substance in acute phase sera from various diseases that reacted with C carbohydrate represented a general reaction of the tissues to injury. The C-reactive protein, as it came to be called, was finally isolated as a highly purified, immunologically homogeneous protein in 1941 (MacLeod & Avery) and subsequently in crystalline form by McCarty in the same laboratory.

Among studies of general importance that in retrospect may be considered as somewhat apart from the main themes of Avery's contributions are those dealing with nutrition and metabolism of pneumococcus and *Haemophilus influenzae* that appeared between 1918 and 1925. With G. E. Cullen he studied acid production of pneumococcus upon growth in various media as well as the autolytic enzyme system. In collaboration with Thjötta it was shown that strains of the influenza bacillus require two vitamin-like factors for growth, the X and V factors, later identified by others as haem and cozymase. With H. J. Morgan he studied various stimulating and inhibiting factors related to growth of pneumococcus, and with J. M. Neill published a series of papers on its intermediary metabolism.

Avery's bibliography lists only three papers on the subject of haemolytic streptococci. One of these, published in 1917 in collaboration with A. R. Dochez and R. C. Lancefield on antigenic composition, set the pattern for the subsequent brilliant contributions of Lancefield and her associates to this subject. There is little doubt but that the example set by the antigenic analysis of pneumococci, and Avery's close though informal relationship for almost 35 years to the studies carried on by Lancefield, contributed greatly to present understanding of the antigenic components of streptococci and their relation to virulence and immunity.

The remarkable report of F. Griffith in 1928 on type transformation of pneumococci formed the starting point for many of the important studies emanating from Avery's laboratory for the next 18 years. In Griffith's ingenious experiments type transformation was achieved by the subcutaneous injection of mice with unencapsulated R cells derived from a particular pneumococcal type together with heat-killed, encapsulated or S cells of homologous or heterologous type. Shortly thereafter, M. H. Dawson and R. H. P. Sia, in Avery's laboratory, succeeded in demonstrating type transformation *in vitro*, and Alloway in 1932 made the next step by preparing cell-free extracts that caused type transformation. Although intensive investigation was continued over the succeeding years no further publications appeared until 1944 (Avery, MacLeod & McCarty) when the evidence was reported which demonstrated that the hereditary changes induced by transformation reactions in pneumococci depend upon the presence of highly polymerized deoxyribonucleic acid (DNA), and by inference that deoxyribonucleic acids are bearers of genetic specificity. The biological specificity of DNA, hitherto unsuspected, was established.

It is worthy of note that at the time this very significant contribution was published Avery had already reached the age of 67, and that he continued for several years afterwards to make contributions of importance to this field.

The respect and affection in which Avery was held by his colleagues was unbounded. Throughout most of his career at the Rockefeller Institute he was known to all as the Professor, or more familiarly, Fess. Although Avery had lived in the United States for many years he did not obtain citizenship until 1917 and was inducted into the U.S. Army as a private, while many of his professional associates at the Rockefeller Institute became officers. He was fond of recounting his experiences of that period of World War I when as a private he conducted classes in the bacteriology of acute infections for Army officers. It was they who dubbed him 'the Professor'. This was his only contact with formal classroom teaching. His influence was exerted rather on individuals who came to work in his laboratory or less directly on those who were members of other departments of the closely knit staff of the Rockefeller Hospital.

His approach to a new assistant was diffident. For a time the recent arrival saw little of the Professor. In some this resulted in a sense of frustration at not being caught up immediately in the scientific life of the active department around them or being made a part of a current problem. Avery did not assign his associates to problems. His approach was indirect and at times seemed excruciatingly slow. After a week or two in the laboratory Avery commonly would invite the new assistant into his tiny personal laboratory that for many years consisted of a former ward kitchen to which the barest bacteriological necessities had been added. A morning or afternoon would be spent in describing the lore of pneumococcus and in tracing the development of knowledge, the problems in which the department was currently concerned and those in which it had an interest. These soliloquies, prose masterpieces of high polish, were widely known as 'Red Seal Records', and Avery was prone to repeat them as he sensed the necessity. If the candidate showed interest and began to read and work under his own steam, he was counselled and aided. A minimum of technical assistance was provided and one swam or sank because of one's own efforts or the lack of them. Avery placed emphasis solely on individual initiative and spurned team projects, or research by squads, as it has been called lately. This method may seem wasteful in the present climate of organized investigation; however, it resulted in a body of work that has few modern parallels in scope and originality. In addition it was productive of a group of graduates who have exerted an influence on medical teaching and biological investigation far out of proportion to their number. In 1946 Avery, with characteristic understatement, on the occasion of his acceptance of the Kober Medal of the Association of American Physicians, attributed his inspiration and guidance to Rufus Cole and ascribed the success of his laboratory to the fact that 'Cole picked these men and all I had to do was pick their brains'.

From the beginning of his career at the Rockefeller Hospital in 1913 until his retirement, save for a 2-year period between 1919 and 1921, Avery and A. R. Dochez were intimately associated, at first as active scientific collaborators on the biological activity of the pneumococcal polysaccharides and later as scientific confidants who shared bachelor quarters for many years. What the great scientific contributions of each owe to the other cannot be disentangled, nor should they be, since ideals and practice coincided so closely. Avery, in making the presentation of the Kober Medal to Dochez in 1949 had the following to say, 'Throughout his studies there is unique continuity of thought centering in the dominant problem of acute respiratory diseases. The results of his work are not random products of chance observation. They are the fruits of years of wise reflection, objective thinking and thoughtful experimentation. I have never seen his laboratory desk piled high with Petri dishes and bristling with test tubes like a forest wherein the trail ends and the searcher becomes lost in dense thickets of confused thought. I have never seen him so busy taking something out of one tube and putting it into another there was no time to think of why he was doing it or of what he was actually looking for. I have never known him to engage in purposeless rivalries or competitive research. But often have I seen him sit calmly by, lost in thought, while all around him others with great show of activity were flitting about like particles in Brownian motion; then, I have watched him rouse himself, smilingly saunter to his desk, assemble a few pipettes, borrow a few tubes of media, perhaps a jar of mice, and then do a simple experiment which answered the very question he had been thinking about when others thought he had been idling in aimless leisure.' This paragraph epitomizes also Avery's own approach to investigation and his philosophy of the 'true inwardness of research'.

Colin MacLeod

BIBLIOGRAPHY OF OSWALD THEODORE AVERY

White, B. & Avery, O. T. (1909). The treponema pallidum; observations on its occurrence and demonstration in syphilitic lesions. *Arch. intern. Med.* 3, 411.

Potter, N. B. & Avery, O. T. (1910). Opsonins and vaccine therapy. In *Mod. Treatment*, 1, 515. Philadelphia and New York: Hare.

White, B. & Avery, O. T. (1910). Observations on certain lactic acid bacteria of the so-called Bulgaricus type. *Zbl. Bakt.* (2. *Abt.*), 25, 161.

Ager, L. C. & Avery, O. T. (1910). A case of influenza meningitis. *Arch. Pediat.* 32, 284.

White, B. & Avery, O. T. (1910). Concerning the bacteremic theory of tuberculosis. *J. med. Res.* 23, 95.

White, B. & Avery, O. T. (1912). The action of certain products obtained from the tubercle bacillus. A. Cleavage products of tuberculo-protein obtained by the method of Vaughan. Communication I. The poisonous substance. *J. med. Res.* **26**, 317.

Avery, O. T. & Lyall, H. W. (1913). Concerning secondary infection in pulmonary tuberculosis. *J. med. Res.* **28**, 111.

White, B. & Avery, O. T. (1913). Some immunity reactions of edestin. The biological reactions of the vegetable proteins. III. *J. infect. Dis.* **13**, 103.

North, C. E., White, B. & Avery O. T. (1914). A septic sore throat epidemic in Cortland and Homer, N.Y. *J. infect. Dis.* **14**, 124.

Dochez, A. R. & Avery, O. T. (1915). Varieties of pneumococcus and their relation to lobar pneumonia. *J. exp. Med.* **21**, 114.

Avery, O. T. (1915). The distribution of the immune bodies occurring in antipneumococcus serum. *J. exp. Med.* **21**, 133.

Dochez, A. R. & Avery, O. T. (1915). The occurrence of carriers of disease-producing types of pneumococcus. *J. exp. Med.* **22**, 105.

Avery, O. T. (1915). A further study on the biologic classification of pneumococci. *J. exp. Med.* **22**, 804.

Dochez, A. R. & Avery, O. T. (1916). Antiblastic immunity. *J. exp. Med.* **23**, 61.

Avery, O. T., Chickering, H. T., Cole, R. & Dochez, A. R. (1917). *Acute lobar pneumonia; prevention and serum treatment.* Monographs of the Rockefeller Institute for Medical Research. No. 7. New York.

Dochez, A. R. & Avery, O. T. (1917). Soluble substance of pneumococcus origin in the blood and urine during lobar pneumonia. *Proc. Soc. exp. Biol., N.Y.* **14**, 126.

Dochez, A. R. & Avery, O. T. (1917). The elaboration of specific soluble substance by pneumococcus during growth. *J. exp. Med.* **26**, 477. *Trans. Ass. Amer. Phycns*, 1917, **32**, 281.

Avery, O. T. (1918). Determination of types of pneumococcus in lobar pneumonia: A rapid cultural method. *J. Amer. med. Ass.* **70**, 17.

Dernby, K. G. & Avery, O. T. (1918). The optimum hydrogen ion concentration for the growth of pneumococcus. *J. exp. Med.* **28**, 345.

Avery, O. T. (1918). A selective medium for *B. influenzae*. Oleate-hemoglobin agar. *J. Amer. med. Assn.* **71**, 2050.

Avery, O. T. & Cullen, G. E. (1919). The use of the final hydrogen ion concentration in differentiation of streptococcus haemolyticus of human and bovine types. *J. exp. Med.* **29**, 215.

Dochez, A. R., Avery, O. T. & Lancefield, R. C. (1919). Studies on the biology of streptococcus. I. Antigenic relationships between strains of streptococcus haemolyticus. *J. exp. Med.* **30**, 179.

Avery, O. T. & Cullen, G. E. (1919). Hydrogen ion concentration of cultures of pneumococci of the different types in carbohydrate media. *J. exp. Med.* **30**, 359.

Avery, O. T., Dochez, A. R. & Lancefield, R. C. (1919). Bacteriology of streptococcus hemolyticus. *Ann. Otol., etc., St Louis*, **28**, 350.

Avery, O. T. & Cullen, G. E. (1920). Studies on the enzymes of pneumococcus. I. Proteolytic enzymes. *J. exp. Med.* **32**, 547.

Avery, O. T. & Cullen, G. E. (1920). Studies on the enzymes of pneumococcus. II. Lipolytic enzymes: esterase. *J. exp. Med.* **32**, 571.

Avery, O. T. & Cullen, G. E. (1920). Studies on the enzymes of pneumococcus. III. Carbohydrate-splitting enzymes: invertase, amylase, and inulase. *J. exp. Med.* **32**, 583.

Thjötta, T. & Avery, O. T. (1921). Studies on bacterial nutrition. II. Growth accessory substances in the cultivation of hemophilic bacilli. *J. exp. Med.* **34**, 97.

Thjötta, T. & Avery, O. T. (1921). Studies on bacterial nutrition. III. Plant tissue, as a source of growth accessory substances in the cultivation of *Bacillus influenzae*. *J. exp. Med.* **34**, 455.

THJÖTTA, T. & AVERY, O. T. (1921). Growth accessory substances in the nutrition of bacteria. *Proc. Soc. exp. Biol., N.Y.* **18**, 197.

AVERY, O. T. & MORGAN, H. J. (1921). The effect of the accessory substances of plant tissue upon growth of bacteria. *Proc. Soc. exp. Biol., N.Y.* **19**, 113.

HEIDELBERGER, M. & AVERY, O. T. (1923). Soluble specific substance of pneumococcus. *J. exp. Med.* **38**, 73.

AVERY, O. T. & HEIDELBERGER, M. (1923). Immunological relationships of cell constituents of pneumococcus. *J. exp. Med.* **38**, 81.

HEIDELBERGER, M. & AVERY, O. T. (1923). The specific soluble substance of pneumococcus. *Proc. Soc. exp. Biol., N.Y.* **20**, 434.

AVERY, O. T. & HEIDELBERGER, M. (1923). Immunological relationships of cell constituents of pneumococcus. *Proc. Soc. exp. Biol., N.Y.* **20**, 435.

AVERY, O. T. & CULLEN, G. E. (1923). Studies on the enzymes of pneumococcus. IV. Bacteriolytic enzyme. *J. exp. Med.* **38**, 199.

AVERY, O. T. & MORGAN, H. J. (1923). Studies on bacterial nutrition. IV. Effect of plant tissue upon growth of pneumococcus and streptococcus. *J. exp. Med.* **38**, 207.

AVERY, O. T. & MORGAN, H. J. (1924). The occurrence of peroxide in cultures of pneumococcus. *J. exp. Med.* **39**, 275.

AVERY, O. T. & MORGAN, H. J. (1924). Studies on bacterial nutrition. V. The effect of plant tissue upon growth of anaerobic bacilli. *J. exp. Med.* **39**, 289.

MORGAN, H. J. & AVERY, O. T. (1924). Growth-inhibitory substances in pneumococcus cultures. *J. exp. Med.* **39**, 335.

AVERY, O. T. & NEILL, J. M. (1924). Studies on oxidation and reduction by pneumococcus. I. Production of peroxide by anaerobic cultures of pneumococcus on exposure to air under conditions not permitting active growth. *J. exp. Med.* **39**, 347.

AVERY, O. T. & NEILL, J. M. (1924). Studies on oxidation and reduction by pneumococcus. II. The production of peroxide by sterile extracts of pneumococcus. *J. exp. Med.* **39**, 357.

AVERY, O. T. & NEILL, J. M. (1924). Studies on oxidation and reduction by pneumococcus. III. Reduction of methylene blue by sterile extracts of pneumococcus. *J. exp. Med.* **39**, 543.

AVERY, O. T. & NEILL, J. M. (1924). Studies on oxidation and reduction by pneumococcus. IV. Oxidation of hemotoxin in sterile extracts of pneumococcus. *J. exp. Med.* **39**, 745.

NEILL, J. M. & AVERY, O. T. (1924). Studies on oxidation and reduction by pneumococcus. V. The destruction of oxyhemoglobin by sterile extracts of pneumococcus. *J. exp. Med.* **39**, 757.

HEIDELBERGER, M. & AVERY, O. T. (1924). The soluble specific substance of pneumococcus. Second paper. *J. exp. Med.* **40**, 301.

NEILL, J. M. & AVERY, O. T. (1924). Studies on oxidation and reduction by pneumococcus. VI. The oxidation of enzymes in sterile extracts of pneumococcus. *J. exp. Med.* **40**, 405.

NEILL, J. M. & AVERY, O. T. (1924). Studies on oxidation and reduction by pneumococcus. VII. Enzyme activity of sterile filtrates of aerobic and anaerobic cultures of pneumococcus. *J. exp. Med.* **40**, 423.

NEILL, J. M. & AVERY, O. T. (1925). Studies on oxidation and reduction by pneumococcus. VIII. Nature of oxidation-reduction systems in sterile pneumococcus extracts. *J. exp. Med.* **41**, 285.

AVERY, O. T. & MORGAN, H. J. (1925). Immunological reactions of isolated carbohydrate and protein of pneumococcus. *J. exp. Med.* **42**, 347.

AVERY, O. T. & NEILL, J. M. (1925). The antigenic properties of solutions of pneumococcus. *J. exp. Med.* **42**, 355.

AVERY, O. T. & HEIDELBERGER, M. (1925). Immunological relationships of cell constituents of pneumococcus. *J. exp. Med.* **42**, 367.

HEIDELBERGER, M., GOEBEL, W. F. & AVERY, O. T. (1925). The soluble specific substance of a strain of Friedländer bacillus. *Proc. Soc. exp. Biol., N.Y.* **23**, 1.

AVERY, O. T., HEIDELBERGER, M. & GOEBEL, W. F. (1925). Immunological behavior of the 'E' strain of Friedländer bacillus and its soluble specific substance. *Proc. Soc. exp. Biol., N.Y.* **23**, 2.

HEIDELBERGER, M., GOEBEL, W. F. & AVERY, O. T. (1925). The soluble specific substance of a strain of Friedländer's bacillus. Paper I. *J. exp. Med.* **42**, 701.

AVERY, O. T., HEIDELBERGER, M. & GOEBEL, W. F. (1925). The soluble specific substance of Friedländer's bacillus. Paper II. Chemical and immunological relationships of pneumococcus type II and of a strain of Friedländer's bacillus. *J. exp. Med.* **42**, 709.

HEIDELBERGER, M., GOEBEL, W. F. & AVERY, O. T. (1925). The soluble specific substance of pneumococcus. Paper III. *J. exp. Med.* **42**, 727.

GOEBEL, W. F. & AVERY, O. T. (1927). The soluble substance of Friedländer's bacillus. III. On the isolation and properties of the specific carbohydrates from Types A and C Friedländer bacillus. *J. exp. Med.* **46**, 601.

AVERY, O. T. & TILLETT, W. S. (1929). Anaphylaxis with the type-specific carbohydrates of pneumococcus. *J. exp. Med.* **49**, 251.

GOEBEL, W. F. & AVERY, O. T. (1929). A study of pneumococcus autolysis. *J. exp. Med.* **49**, 267.

HEIDELBERGER, M., AVERY, O. T. & GOEBEL, W. F. (1929). A 'soluble specific substance' derived from gum arabic. *J. exp. Med.* **49**, 847.

GOEBEL, W. F. & AVERY, O. T. (1929). Chemo-immunological studies on conjugated carbohydrate-proteins. I. The synthesis of *p*-aminophenol *β*-glucoside, *p*-aminophenol *β*-galactoside, and their coupling with serum globulin. *J. exp. Med.* **50**, 521.

AVERY, O. T. & GOEBEL, W. F. (1929). Chemo-immunological studies on conjugated carbohydrate-proteins. II. Immunological specificity of synthetic sugar-protein antigens. *J. exp. Med.* **50**, 533.

TILLETT, W. S., AVERY, O. T. & GOEBEL, W. F. (1929). Chemo-immunological studies on conjugated carbohydrate-proteins. III. Active and passive anaphylaxis with synthetic sugar-proteins. *J. exp. Med.* **50**, 551.

TILLETT, W. S., GOEBEL, W. F. & AVERY, O. T. (1930). Chemical and immunological properties of a species-specific carbohydrate of pneumococci. *J. exp. Med.* **52**, 895.

AVERY, O. T. & DUBOS, R. (1930). The specific action of a bacterial enzyme on pneumococci of type III. *Science*, **72**, 151.

AVERY, O. T. & DUBOS, R. (1931). The specific action of a bacterial enzyme on type III pneumococci. *Trans. Ass. Amer. Phycns*, **46**, 216.

AVERY, O. T. & DUBOS, R. (1931). The protective action of a specific enzyme against type III pneumococcus infection in mice. *J. exp. Med.* **54**, 73.

DUBOS, R. & AVERY, O. T. (1931). Decomposition of the capsular polysaccharide of pneumococcus type III by a bacterial enzyme. *J. exp. Med.* **54**, 51.

GOEBEL, W. F. & AVERY, O. T. (1931). Chemo-immunological studies on conjugated carbohydrate-proteins. IV. The synthesis of the *p*-aminobenzyl ether of the soluble specific substance of type III pneumococcus and its coupling with protein. *J. exp. Med.* **54**, 431.

AVERY, O. T. & GOEBEL, W. F. (1931). Chemo-immunological studies on conjugated carbohydrate-proteins. V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of type III pneumococcus with foreign protein. *J. exp. Med.* **54**, 437.

GOEBEL, W. F., BABERS, F. H. & AVERY, O. T. (1932). Chemo-immunological studies on conjugated carbohydrate-proteins. VI. The synthesis of *p*-aminophenol *α*-glucoside and its coupling with protein. *J. exp. Med.* **55**, 761.

Avery, O. T., Goebel, W. F. & Babers, F. H. (1932). Chemo-immunological studies on conjugated carbohydrate proteins. VII. Immunological specificity of antigens prepared by combining *a*- and *b*-glucosides of glucose with proteins. *J. exp. Med.* **55**, 769.

Avery, O. T. (1932). The role of specific carbohydrates in pneumococcus infection and immunity. *Ann. intern. Med.* **6**, 1.

Goodner, K., Dubos, R. & Avery, O. T. (1932). The action of a specific enzyme upon the dermal infection of rabbits with type III pneumococcus. *J. exp. Med.* **55**, 393.

Avery, O. T. (1933). Chemo-immunologische Untersuchungen an Pneumokokken-Infektion und-Immunität. *Naturwissenschaften*, **21**, 777.

Avery, O. T. & Goebel, W. F. (1933). Chemo-immunological studies on soluble specific substance of pneumococcus. I. The isolation and properties of acetyl polysaccharide of pneumococcus type I. *J. exp. Med.* **58**, 731.

Goebel, W. F., Babers, F. H. & Avery, O. T. (1934). Chemo-immunological studies on conjugated carbohydrate proteins. VIII. The influence of the acetyl group on the specificity of hexoside-protein antigens. *J. exp. Med.* **60**, 85.

Goebel, W. F., Avery, O. T. & Babers, F. H. (1934). Chemo-immunological studies on conjugated carbohydrate proteins. IX. The specificity of antigens prepared by combining the *p*-aminophenol glycosides of disaccharides with protein. *J. exp. Med.* **60**, 599.

Francis, T., Terrell, E. E., Dubos, R. & Avery, O. T. (1934). Experimental type III pneumococcus pneumonia in monkeys. II. Treatment with an enzyme which decomposes the specific capsular polysaccharide of pneumococcus type III. *J. exp. Med.* **59**, 641.

Abernethy, T. J. & Avery, O. T. (1941). The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. *J. exp. Med.* **73**, 173.

MacLeod, C. M. & Avery, O. T. (1941). The occurrence during acute infections of a protein not normally present in the blood. II. Isolation and properties of the reactive protein. *J. exp. Med.* **73**, 183.

MacLeod, C. M. & Avery, O. T. (1941). The occurrence during acute infections of a protein not normally present in the blood. III. Immunological properties of the C-reactive protein and its differentiation from normal blood proteins. *J. exp. Med.* **73**, 191.

Avery, O. T. (1944). Karl Landsteiner. Born 14 June 1868. Died 26 June 1943. *J. Path. Bact.* **56**, 592.

Avery, O. T., MacLeod, C. M. & McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. exp. Med.* **79**, 137.

McCarty, M. & Avery, O. T. (1946). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonuclease on the biological activity of the transforming substance. *J. exp. Med.* **83**, 89.

McCarty, M. & Avery, O. T. (1946). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. III. An improved method for the isolation of the transforming substance and its application to pneumococcus types II, III, and VI. *J. exp. Med.* **83**, 97.

Avery, O. T. (1946). Acceptance of the Kober medal award. *Trans. Ass. Amer. Phycns*, **59**, 43.

McCarty, M., Taylor, H. E. & Avery, O. T. (1946). Biochemical studies of environmental factors essential in transformation of pneumococcal types. In *Cold Spr. Harb. Symp. quant. Biol.* **11**, 177.

OSWALD THEODORE AVERY

Awards

John Phillips Memorial Award, American College of Physicians, 1932.
Paul Ehrlich Medal, 1932.
Copley Medal, Royal Society of London, 1945.
Kober Medal, Association of American Physicians, 1945.
Passano Foundation Award, 1949.

Honorary Degrees

Colgate University, Sc.D., 1921.
McGill University, LL.D., 1935.
New York University, Sc.D., 1947.
Rutgers University, Sc.D., 1953.

Membership in National Academies

National Academy of Sciences of the United States of America.
Foreign Member, Royal Society of London.
Honorary Member, Société Philomathique de Paris.
Honorary Member, Académie Royale de Médecine de Belgique.

Furness, G. & Rowley, D. (1957). *J. gen. Microbiol.* **17**, 550–561

The Presence of the Transmissible Agent F in Non-Recombining Strains of *Escherichia coli*

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SUMMARY: A proportion of wild-type strains of *Escherichia coli* unable to recombine with *E. coli* K12 F− mutants can 'infect' them with the sex-differentiating character known as F. Of these strains, not all infect the same *E. coli* K12 F− mutants and the ease of transfer of F varies among them, suggesting that there are different F agents. This is supported by the finding that there are great variations in the transfer of unselected markers in crosses in which the only known difference is the origin of the F factor possessed by the F+ parent.

Colicinogenicity and F are frequently associated and all of nine strains producing colicin I possess F, but they are not identical, since the two properties have been separated by treatment with cobalt chloride.

Recombination between mutants of *Escherichia coli* K12 appears to require cell to cell contact. There are differences between the parent cells in that one donates (F+) and one accepts (F−) genetic material (Hayes, 1953). The F+ character (or Hfr) must be present in at least one of the parent strains for a cross to be fertile (Lederberg, Cavalli & Lederberg, 1952). One of the most important characteristics of this F+ character is that of being infective, that is, a high proportion of an F− strain can be made F+ by growing an F+ and F− strain together for a short time, i.e. 1–2 hr. (Cavalli, Lederberg & Lederberg, 1953). Once acquired the F+ character is inherited. In spite of this infective nature, which suggests a similarity to phage, it has not been possible to demonstrate the F+ character in the absence of bacterial cells. The possibility that the F agent might be of phage or prophage nature obviously deserves consideration.

Whilst it is certain that the F character is necessary for recombination to occur in *Escherichia coli* K12, it is by no means certain that possession of a transmissible F agent is sufficient to enable a strain to recombine. Cavalli *et al.* (1953) searched for transmissible F agents in *E. coli* and *Salmonella typhimurium* strains, using its infective property for an *E. coli* K12 F− mutant as the criterion of its presence, and failed to find F in any strains infertile with K12 mutants.

Some *Escherichia coli* and other enteric organisms are colicinogenic, that is they produce various colicins (Gratia, 1925; Fredericq, 1948). This property, like the F+ character, can often be transferred to other non-colicinogenic strains by growing them with the colicinogenic strain. Using mutants of K12, Fredericq (1954*b*) has shown that F has to be present in either the colicinogenic (donor) or non-colicinogenic (acceptor) strain for the transfer to take place. Moreover, he has reported the transfer of colicinogenicity to an

F− mutant of K12 from a strain of *Shigella sonnei* (Fredericq, 1954*a*) which so far as we know is not interfertile with K12. This suggested to us that this Shigella strain might contain an F agent without itself being fertile.

More recently, further data have been produced by Jacob & Wollman (1955). In order to account for the extreme rapidity and great efficiency of transfer of the F agent they have postulated that it is attached near to that part of the chromosome (O) which first enters the F− parent during the process of chromosome transfer and that there is a point 'R' on the chromosome situated between F and the next known marker where the chromosome preferentially breaks. According to this theory, F transfer is an early and necessary step in the process of recombination.

It would seem likely that cells possessing F and able to transfer it to F− cells might reasonably be expected to undergo recombination for other markers also. For this reason, we have investigated the possibility implied by Fredericq's work that F may occur in strains that cannot be shown to recombine by the usual methods. This supposition was confirmed and extended, in that six out of thirty randomly chosen wild-type strains of *Escherichia coli* were found to carry F agents, though they were not demonstrably fertile with K12.

METHODS

Cultures

All cultures were grown at 37°.

Wild-type strains of Escherichia coli. These were randomly isolated during the routine examination of specimens obtained from patients, no two cultures being obtained from the same person. With the exception of *E. coli* I_4, which was of an 'intermediate' type, the wild strains were indole and methyl-red positive, V.P. negative, and unable to utilize citrate as the sole source of carbon. They were sensitive to 500 μg. streptomycin/ml. With one exception, those transferring F differed from each other either in their biochemical reactions or in the colicins they produced (see Table 1).

Strains of Escherichia coli *K12.* The following auxotrophic mutants were used: Strain 58.161—a methionine-requiring mutant (M^-) fermenting lactose, arabinose, galactose, maltose, xylose and mannitol (lac^+, $arab^+$, $gal_5{}^+$, mal^+, xyl^+, mtl^+). Strain W677—auxotrophic for threonine, leucine and thiamine (T^-, L^-, $B_1{}^-$), and not fermenting lactose, arabinose, galactose, maltose, xylose or mannitol (lac^-, $arab^-$, $gal_5{}^-$, mal^-, xyl^-, mtl^-).

Both streptomycin-sensitive and -resistant mutants of F+ and F− strains were used. Strains 58.161 $S^R(1)F-$ and 58.161 $S^R(2)F-$ were two independent streptomycin-resistant mutants.

Colicin-sensitive indicator strains. Escherichia coli B and *Shigella dispar* P15, kindly supplied by Professor P. Fredericq, were used as indicator strains for colicinogenicity; the K12 mutant W677 S^RF- was also used.

Media. The formula of Davis & Mingioli (1950) was used for the preparation of minimal medium and that of Lederberg (1950) for eosin-methylene-blue sugar-containing media. The minimal medium was supplemented with

Table 1. *Differential characters of wild strains of* Escherichia coli *transferring F*

Culture of *E. coli*	Colicins produced	Fermentation of					
		Dulcitol	Galactose	Lactose	Salicin	Sucrose	Xylose
A	—	$-\ -_3$	$A_2\ -_3$	A_1G_1	$-\ -_3$	$-\ -_3$	$A_2\ -_3$
H	I	A_2G_2	A_2G_2	A_1G_1	A_3G_3	$-\ -_3$	A_1G_1
J	V, B	A_2G_2	A_1G_1	A_1G_1	A_2G_2	$-\ -_4$	A_1G_1
L	—	A_1G_1	A_1G_1	A_1G_1	A_1G_1	A_1G_1	A_1G_1
O	B	A_2G_2	A_1G_1	A_1G_1	A_2G_2	$-\ -_4$	A_1G_1
R	I, B	A_2G_2	A_1G_1	A_1G_1	$-\ -_4$	$-\ -_4$	A_1G_1
U	I*	A_2G_2	A_1G_1	A_1G_1	$-\ -_3$	$-\ -_3$	A_1G_1
I_1	I	A_2G_2	A_1G_1	A_1G_1	A_2G_2	$-\ -_6$	A_1G_1
I_2	I	A_1G_1	A_1G_1	A_1G_1	A_2G_3	$-\ -_6$	A_1G_1
I_3	I	A_1G_1	A_1G_1	A_1G_1	A_2G_3	$-\ -_6$	A_1G_1
I_4†	I	$-\ -_3$	A_1G_1	A_2G_2	A_2G_2	$-\ -_3$	A_1G_1
I_5	I	A_1G_1	A_1G_1	A_1G_1	A_2G_2	A_1G_1	A_1G_1
I_6	I	$-\ -_4$	A_1G_1	A_1G_1	A_2G_2	$-\ -_4$	A_1G_1

A, denotes acid; G, gas; —, negative; numeral suffix, days incubated to obtain result; *, probably two others; †, intermediate type.

All strains ferment arabinose, glucose, maltose, and mannitol within 24 hr. with production of acid and gas.

20 μg. thiamine/ml. when necessary. All streptomycin media contained 500 μg. streptomycin/ml.

Transfer of F. Although transfer of F to *Escherichia coli* B occurs only with difficulty, De Haan (1954) found *E. coli* B could be infected with F from K12 mutants by growing the two strains together in broth. The following adaptation of his method was used. To 100 ml. of nutrient broth were added about 3×10^8 organisms of the wild and the K12 S^RF- tester strains (58.161 and W677). After incubation of the mixture for 24 hr. at 37° it was diluted approximately 1/20,000 into fresh broth and incubated for a further 24 hr. Serial transfer of the mixture was repeated at least three times. The K12 S^R strain was recovered by overnight incubation of 20 ml. of streptomycin broth inoculated with 1/200 ml. of the mixed culture. Since the K12 S^R mutant was originally F— it could not recombine with an alternative F— strain unless it had become F+ during serial transfer with the wild *E. coli* strain. This culture recovered in streptomycin broth was tested for infection with F by attempting recombination with an F— strain as follows: 4 ml. of the streptomycin broth culture was mixed with an equal volume of the K12 S^RF- mutant having complementary auxotrophic characters; for example, when 58.161 S^R was tested for the possession of F it was mixed with W677 S^RF-. The mixture and controls of 5 ml. of each parent separately were washed twice in minimal salts (Davis & Mingioli, 1950), resuspended in 0·2 ml. and spread on plates containing streptomycin minimal medium alone when the F— parent was M^-, and supplemented with thiamine when the F— parent was TLB_1^-. Since in these experiments both the K12 strain under test and the complementary K12 F— strain were streptomycin-resistant the presence of streptomycin in the minimal medium should not diminish the yield of prototrophic recombinants; but would ensure that any surviving cells of the wild-type strain under test would not form colonies (since all the wild-type strains were streptomycin-sensitive).

Replica recombination technique. Each F + parent was spread over a circular area about 0·5 cm. in diameter on a nutrient agar plate, which was incubated for 5–6 hr. and then replica-plated by a velveteen pad on to a lawn plate of streptomycin minimal or a streptomycin minimal thiamine medium seeded with about 3×10^9 washed cells of the F – parent.

Recombination in broth. 4 ml. of an overnight culture of the wild strain of *Escherichia coli* and the K12 S^RF – mutant were mixed and incubated together for 8–24 hr. The mixture, and 5 ml. of each parent, as controls, were washed twice in minimal salts, resuspended in 0·2 ml. and spread on plates containing streptomycin minimal medium alone when the F – parent was M^- and supplemented with thiamine when the F – parent was TLB^-.

A representative sample of the colonies appearing from recombination experiments was purified and analysed for the transfer of unselected markers by the technique of Clowes & Rowley (1954) to confirm that they were recombinants.

Testing for colicinogenicity

The strain of *Escherichia coli* to be tested was plated to give single colonies on nutrient agar. After 24 hr. incubation the culture was sterilized by subjecting it to chloroform vapour for 30 min. (Fredericq, 1948) and layered with nutrient agar. The surface was then spread with a suspension of the indicator strain, dried and incubated overnight at 37°.

RESULTS

Transfer of F

Thirty wild-type strains of *Escherichia coli* were screened for their ability to transfer F to the K12 mutants 58.161 S^R(1)F – and to W677 S^RF – by successive transfer of the mixed culture in broth. Although the K12 mutant 58.161 S^R(1) was recovered in every case by inoculating the mixture into streptomycin broth, W677 S^R was only recovered from 14 of the 30 mixtures. Subsequently, 14 of the 16 wild-type strains contained in mixtures from which W677 S^R could not be recovered were shown to produce a colicin to which W677 S^RF – was sensitive, this perhaps explains the elimination of W677 S^R F – in some of these mixtures. The ability of 7 of the 30 recovered 58.161 S^R(1) tester strains to recombine with another F – strain indicated that 7 of the 30 wild strains of *E. coli*, A, H, J, L, O, R and U were able to transfer F to 58.161 S^R(1)F – (see Table 2), but of the 14 mixtures in which the W677 tester strain was recovered only one, strain A, gave a positive result. Control recombination plates seeded with the recovered K12 tester strain alone never grew colonies, confirming that those obtained in the crosses were not due to recombination between the wild and F – strains during serial transfer, nor to mutation of the wild strain to streptomycin-resistance during recovery of the *E. coli* K12 S^RF – strain from the mixture. The seven *E. coli* strains found to possess this F+ character were tested for fertility with K12 by the 'recombination in broth' method; *E. coli* strain A alone recombined with 58.161 S^R(1) F−. Thus 6 wild strains of *E. coli*, H, J, L, O, R and U, could transfer fertility to a K12 F− mutant without themselves being fertile, with the K12 mutants.

Table 2. *Differences in the number of recombinants obtained under standard conditions from matings of* Escherichia coli *W677 S^RF- with* E. coli 58.161 S^R(1) *infected with F by contact with various wild strains of* E. coli

Culture of *E. coli* used to transfer F to 58.161 S^R(1)	No. of recombinants
A	250–500
H	100–1000
J	10–50
L	10–50
O	10–50
R	50–100
U	10–50
Control 58.161 S^{R}F+	1000
58.161 S^R(1)F−	0

The wild strains differed significantly in their ability to transfer F to the two K12 F− mutants (see Table 3). Moreover, these K12 mutants have unpredictable differences in their ability to become infected with the F+ character, although they were derived from the same parent strain. Differences in the ability of K12 mutants to become infected with the F+ character were confirmed when attempts were made to infect a third K12 mutant, 58.161 S^R(2)F−, with the F of the wild strains (see Table 3).

Table 3. *Differences in the ability of wild strains of* Escherichia coli *to transfer the F+ character and colicinogenicity to* E. coli 58.161 S^R(1)$F-$, 58.161 S^R(2)$F-$ *and W677 S^RF-*

Wild strains of *E. coli*	K12 acceptor strain					
	58.161 S^R(1)F−		58.161 S^R(2)F−		W677 S^{R}F−	
	Character transferred					
	F+	colicinogenesis	F+	colicinogenesis	F+	colicinogenesis
A*	+	NC	−	NC	+	NC
H	+	+	+	+	+	+
J	+	−	−	−	−	−
L	+	NC	−	NC	−	NC
O	+	−	−	−	−	−
R	+	+†	−	−	−	−
U	+	+†	+	−	−	−
I$_1$	+	+	−	+	−	+
I$_2$	+	−	−	−	−	−
I$_3$	+	−	−	+	−	+
I$_4$	−	−	+	+	−	+
I$_5$	−	+	+	+	−	+
I$_6$	−	−	−	+	+	+

* Can recombine with *E. coli* 58.161 S^R(1)F− *c.* 1 in 10)^8.

−, Negative result; +, positive result; NC, wild strain not colicinogenic; † indirect evidence, see text.

A minimum of three successive transfers was empirically chosen when screening the 30 wild strains for their ability to transfer F. It was subsequently found that wild strains varied considerably in the minimal time of mixed growth required to transfer detectable fertility to the K12 tester strains.

Stability of F+ state

The streptomycin-broth cultures of 58.161 $S^R(1)$ which had been recovered from a mixture with *Escherichia coli* H and which presumably contained F+ organisms since it recombined with W677 S^RF-, was plated out on nutrient agar. Single colonies found to be F+ on first isolation lost this character on subculture; indicating that the F agent of *E. coli* H was unstable in strain 58.161 $S^R(1)$. Some F+ clones were therefore plated out and single colonies tested for fertility by replica recombination. Those clones producing the largest number of recombinants were again plated out, and the colonies again tested by the replica recombination method until a stable fertile strain was obtained. A stable strain of 58.161 $S^R(1)$ infected with F from *E. coli* H was thus isolated and labelled 58.161 $S^R(1)F_H$.

This stable strain was then used as F+ donor in further transfer experiments with 58.161 F− and W677 F−. However, on testing single colonies obtained from F+ clones the F state of either 58.161 or W677 was found to be unstable which indicated that the stability of the F+ state of strain 58.161 $S^R(1)$ F_H did not result from an alteration of the F agent obtained from *E. coli* strain H.

In the course of these experiments it had been noted that after exposure to *Escherichia coli* H the colonies of the K12 mutants 58.161 $S^R(1)$, 58.161 F− and W677 F− varied in size from 1 mm. in diameter to the normal 2–3 mm. Sometimes one or more segments of a small colony were of normal size. This suggests that F+ clones when first infected with F could be detected by colony size.

The elimination of Escherichia coli *K12 F− mutants from mixed cultures by* E. coli *H*

When *Escherichia coli* strain H was grown together with W677 S^RF- it was found that the wild strain rapidly came to predominate. The rate of elimination of both 58.161 $S^R(1)F-$ and W677 S^RF- was ascertained by making differential counts of mixtures after varying periods of growth. Mixed cultures of *E. coli* H and W677 S^RF- or 58.161 $S^R(1)F-$ were studied and in the course of about fifty generations the ratio of *E. coli* H to W677 S^RF- was found to increase from 1:3 to 2×10^6:1. Similarly, the ratio of *E. coli* H to 58.161 $S^R(1)F-$ changed from 1:5 to 680:1.

These results suggested possible antagonism and further investigation revealed that *Escherichia coli* H produced a colicin to which K12 mutants were sensitive. Of the 30 wild strains tested for the F+ character 19, including strains J, O, R and U, were found to be colicinogenic; they produced wide zones of inhibition of the K12 mutants W677 and 58.161 after overnight incubation. That is, of the six non-fertile wild strains shown to be able to infect K12 strains with the F agent five were shown to be colicinogenic; the remaining strain, *E. coli* L, was further tested for colicinogenicity after varying periods of incubation using *E. coli* B, *E. coli* W677 and *Shigella dispar* P. 15 as indicator strains, without any evidence of the production of a colicin. Professor P. Fredericq kindly identified the colicins produced by the colicinogenic strains which are enumerated in Table 1. Three of these five strains produce more than one colicin. Three strains produce colicin B (Fredericq, 1948) and three colicin I, and one strain, R, produces both B and I.

Transfer of colicinogenicity

As five of the six wild strains able to transfer F were colicinogenic the transfer of colicinogenicity to the K12 S^RF− mutants was examined by the techniques used for the transfer of F. Colonies of the K12 S^R mutants recovered from the mixtures by plating on streptomycin agar were transferred to a second plate by replica plating and the original streptomycin plate was tested for colicin production. Thus transfer of F and colicinogenicity could be compared.

Of the five colicin-producing strains, H, J, O, R and U, only one, *E. coli* H, transferred colicinogenicity to all the three K12 S^RF− mutants (see Table 3). Transfer of both the F+ and colicinogenic attributes from strain H to 58.161 S^R(1)F− and 58.161 S^R(2)F− occurred within 24 hr., the majority of the colonies being colicinogenic. In contrast, using W677 S^RF−, colicinogenic colonies were rare although all those possessing this attribute were also F+. When colicinogenic colonies of either W677 S^R or 58.161 S^R(1) were plated out only a small proportion of the colonies obtained were colicinogenic, indicating that their colicinogenic property like the F+ state was unstable.

The colicinogenic *Escherichia coli* strains, J, O, R and U could not be shown by the techniques employed to transfer colicinogenicity to the F− tester strains, although they could all transfer F to 58.161 S^R(1)F−, and one of them (*E. coli* U), to 58.161 S^R(2)F− also (see Table 3). Some of the recombinants derived from crosses of W677 S^RF− with 58.161 S^R(1)F− infected with F by contact with *E. coli* R and U were, however, colicinogenic when first isolated, but those in which F was derived from *E. coli* R had lost this property when tested again. As these recombinants were colicinogenic it would be inferred that the F+ parents were also colicinogenic, and the fact that this could not be demonstrated directly shows the inadequacy of the technique used.

The relationship between production of colicin I and the F state

Since the F+ and colicinogenic properties have been shown to involve similarities in transfer, particularly with respect to time of transfer and stability in the new host, the possibility exists that there may be a connexion between these factors. The most competent F+ donor, strain H, produced colicin I only. A search was therefore made amongst a further 70 wild strains for those producing this colicin. Each strain was plated out on two plates of nutrient agar and tested for colicin production by the normal technique using

W677 as a universal indicator strain and 58.161 $S^R(1)F_H$ as the indicator strain which was resistant to colicin I alone. Only those wild strains producing a colicin active on W677 and giving no evidence of action on 58.161 $S^R(1)F_H$ were tested for F. Six such strains were found: *E. coli* I_1, I_2, I_3, I_4, I_5, I_6. All were streptomycin-sensitive and with the exception of *E. coli* I_2 and I_3, were distinguishable from each other by biochemical tests (see Table 1).

These strains were examined for their ability to transfer colicinogenicity and F to K12 mutants. All the six strains could transfer F to one or other of the K12 S^RF− mutants, although there were differences in this respect (see Table 3). Similarly, they varied in their ability to transfer colicinogenicity (see Table 3). With the one exception of *Escherichia coli* I_2 transfer of fertility was shown to be accompanied by the ability to transfer colicinogenicity. Since both attributes (F+ and colicinogenicity) however were unstable to varying degrees in their new bacterial host, it was not possible to obtain a clear answer as to whether both factors are always transferred together and a different approach had to be used in order to demonstrate that these factors are clearly separate.

Elimination of F and colicinogenicity

An attempt was made to eliminate colicinogenicity by treating cells of 58.161 $S^R(1)F_H$ suspended in minimal salts with varying doses of UV light. Several thousand colonies derived from these cells were tested and all were colicinogenic. Thereafter, use was made of the observation by Hirota (1956) that treatment of K12 F+ mutants with cobalt chloride caused some of the cells to become F−.

Peptone water (1 % 'Evans' peptone, 0·2 % (w/v) glucose, pH 7·4) inoculated with 58.161 $S^R(1)F_H$, which was stable for both colicinogenicity and F, was incubated for 24 hr; cobalt chloride was added to give a final concentration of 0·02 M. The peptone water was re-incubated overnight and plated on nutrient agar containing 0·04 M-sodium citrate; single colonies were tested for colicinogenicity. Out of 450 colonies tested for colicinogenicity two were non-colicinogenic; they were found to be F− also. 147 colonies of an untreated culture of 58.161 $S^R(1)F_H$ were tested for the F+ character; all were positive, indicating the stability of this character. One of 14 colonies derived from a culture treated with cobalt chloride however was F−, but was still colicinogenic. It is therefore possible to separate these two properties.

The effect of F on the transfer of unselected markers

In all recombination experiments involving, as F+ parent, a mutant of K12 infected by a wild strain of *Escherichia coli*, some of the recombinants were examined for the transfer of unselected markers. The results for sugar markers, thiamine-requirement and colicinogenicity for nine crosses are given in Table 4. Although the numbers are small it is evident that there were significant differences in the extent of transfer of these characters to the recombinants, depending on the origin of the F carried by the donor strain. The marked differences in transfer between crosses 3, 5 and 6 suggests that the original wild strains may contain different F agents. This difference was most marked

Table 4. *Transfer of unselected markers by mutants of* Escherichia coli *K*12 *infected with F by wild strains of* E. coli

				Percentage unselected markers transferred to the recombinants							
					Fermentation of						
Crosses	Selecting medium	Selected markers of F+ parent	No. of colonies tested	B_1*	Lac	Arab	Gal	Mal	Xyl	Mtl	Colicinogenesis
(1) *A/58.161 S^R(1)F+ × W677 S^RF−	MMB_1	T.L.	241	29·7	71	45	42	39	25	27	N.C.
(2) A/58.161 S^R(1)F+ × W677 S^RF−	MM	T.L.B.$_1$	72		85	55	55	60	44	44	N.C.
(3) H/58.161 S^R(1)F+ × W677 S^RF−	MMB_1	T.L.	206	13·6	41	18	18	18	16	16	97
(4) H/58.161 S^R(1)F+ × W677 S^RF−	MM	T.L.B.$_1$	114		54	50	50	39	46	45	N.T.
(5) I_1/58.161 S^R(1)F+ × W677 S^RF−	MMB_1	T.L.	48	0	29·2	0	0	0	0	0	14·6
(6) I_2/58.161 S^R(1)F+ × W677 S^RF−	MMB_1	T.L.	40	2·5	5·1	0	0	0	0	0	0
(7) I_3/58.161 S^R(1)F+ × W677 S^RF−	MMB_1	T.L.	49	4·1	23·4	0	0	0	0	0	0
(8) H/W677 S^RF+ × 58.161 S^R(1)F−	MM	M	43		44·3	53·3	46·5	67·5	63·7	72	100
(9) A/W677 S^RF+ × 58.161 S^R(1)F−	MM	M	243		6	23	23	26	28	30	N.C.

* The prefix to the F+ parent denotes the wild strain denoting F. N.C. F+ parent non-colicinogenic; N.T. recombinant not tested for colicinogenesis.

when using as the F donor the wild strain A, which was itself weakly fertile with 58.161 S^R(1)F−. When data from crosses 1 and 3 in Table 4, i.e. A/58.161 S^{R}F+ and H/58.161 S^{R}F+ × W677 S^{R}F−, are analysed in detail by arranging the sugar markers, by trial and error, in the order which accounts for the greatest proportion of recombinants by single cross-overs (Table 5), it is found that in spite of great differences in the total transfer of sugar markers from the F+ parents the same order of markers is obtained, namely, lactose, arabinose, galactose, maltose, xylose and mannitol. These data are insufficient to determine the relative order of xylose and mannitol. This is in partial agreement with the order given by Clowes & Rowley (1954) and in agreement with Cavalli-Sforza & Jinks (1956).

Table 5. *Segregation of sugar markers on mating* Escherichia coli 58.161 *S^R*(1) *infected with F by* E. coli *A or H with* E. coli *W677 S^RF− on minimal medium supplemented with B_1*

Recombinant class						Number of recombinants in class			
						T+L+B_1^- recombinants F+ parent		T+L+B_1^+ recombinants F+ parent	
Lac	Arab	Gal	Mal	Xyl	Mtl	*A 58.161 S^R(1)F+	*H58.161 S^R(1)F+	*A58.161 S^R(1)F+	*H58.161 S^R(1)F+
−	−	−	−	−	−	19	89	6	10
+	−	−	−	−	−	44	39	12	1
+	+	−	−	−	−	9	3	2	2
+	+	+	−	−	−	14	3	4	3
+	+	+	+	−	−	10	8	8	1
+	+	+	+	+	−	3	0	3	0
+	+	+	+	+	+	18	13	19	3
−	−	+	+	+	+	.	.	.	1
−	−	−	+	+	+	2	1	.	.
−	−	−	−	+	+	2	4	1	.
+	+	+	−	−	+	1	.	1	.
+	−	−	+	+	+	5	1	1	.
+	−	−	−	+	+	2	.	.	.
+	−	−	−	−	+	1	1	.	.
−	+	−	−	−	−	3	2	.	1
−	−	+	−	−	−	3	1	.	1
−	−	−	+	−	−	4	3	.	.
−	−	−	−	+	−	1	2	.	.
−	−	−	−	−	+	.	.	1	.
−	+	+	−	−	−	3	.	1	.
−	+	+	+	+	−	.	1	.	.
−	−	−	−	+	+	2	.	1	.
−	+	+	+	+	+	1	.	.	.
+	−	+	+	+	+	3	2	3	.
+	+	−	+	+	+	1	.	1	.
+	+	+	−	+	+	2	2	.	2
+	+	+	−	−	+	.	.	1	1
+	+	+	+	−	+	6	.	.	.
+	+	−	+	−	−	.	.	1	.
+	+	−	−	+	−	2	.	.	.
+	−	+	+	−	−	.	.	1	.
+	−	−	+	+	−	2	.	.	.
+	−	+	+	+	−	.	2	.	.
+	−	−	+	−	−	3	.	1	.
+	−	+	−	−	−	1	.	.	1
+	−	−	+	−	+	1	.	3	.
−	−	+	−	+	−	1	.	.	.
−	+	+	+	−	+	1	.	.	.
−	+	+	−	+	+	.	.	.	1
−	+	−	−	−	+	.	1	1	.
Total recombinants						170	178	72	28

Markers of parent strains of K12 mutant. 58.161: −M$^-$, Lac$^+$, Arab$^+$, Gal$^+$, Mal$^+$, Xyl$^+$, Mtl$^+$. W677: −T$^-$L$^-$.B_1^-, Lac$^-$, Arab$^-$, al$^-$, Mal$^-$, Xyl$^-$, Mtl$^-$.

* Prefix letter denotes the wild strain denoting F.

DISCUSSION

Wild cultures of *Escherichia coli* which would cross with K12 F− are known but it had not previously been shown that infertile strains may contain the F character (Cavalli *et al.* 1953) until a strain was reported by Lederberg & Lederberg (1956) which was infertile with K12 F− mutants yet transferred F. It has now been found that a relatively high proportion of wild strains, infertile according to our tests with the F− strains, can infect them with F. Other factors, besides the possession of F, are therefore involved in the ability of a strain to recombine with K12 auxotrophs to give phototrophic (or B_1-exacting) recombinants.

Individual mutants derived from the same parent can differ in their ability to accept F agents and two independently isolated streptomycin-resistant mutants of 58.161 F− are completely different in this respect. Hayes (1953) similarly has reported that M−F− and TLB_1^-F- mutants of K12 vary in their ability to become infected with the F of K12. A greater proportion of wild strains might have been shown to possess a transmissible F agent if, in the present work, other F− tester strains and more exhaustive techniques had been employed, such as colicin-resistant strains and daily examination for transfer of F.

Differences in the ease of transfer of F from wild strains of *Escherichia coli* to the standard K12 S^RF- mutants suggests that there is more than one F agent. This is supported by the fact that not every wild strain can infect the same F− mutant. After infection the F state is lost spontaneously, a finding previously noted by Lederberg *et al.* (1952) suggesting that these F agents differ from that of K12. Moreover, this suggestion is further strengthened by the recombination data in which there are great variations in the transfer of unselected markers in crosses which only differ, so far as we know, in the F agent carried by the F+ parent. Another F agent which affects gene recombination has also been reported (Bernstein, 1957).

There are several similarities between colicinogenicity and the F+ state in K12. Both are transferred rapidly when donor and acceptor strains are incubated together. Colicin production can be increased by UV irradiation and similarly fertility is increased in recombination after irradiating the F+ parent (Hayes, 1952). The widely used mutants of K12 have never been shown to produce colicins, although this might be due to lack of a suitable indicator strain. Five of the six wild strains of *Escherichia coli* which transfer F in these experiments are able to produce colicins I or B or both. A search for wild types producing the same colicin, i.e. colicin I, as strain H, which had the most easily transmissible F agent, revealed six more strains, all of which were F+ but could not recombine. Thus, in this small series, strains producing colicin I have been shown to be F+. On the other hand, although these two properties are similar in many respects, they are different entities, since it was found that treatment with cobalt chloride could eliminate the F agent without loss of colicinogenicity.

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REFERENCES

BERNSTEIN, H. L. (1957). A new infective factor controlling sex compatibility and gene recombination in *Escherichia coli*. *J. gen. Microbiol.* **16**, iii.

CAVALLI, L. L., LEDERBERG, J. & LEDERBERG, E. M. (1953). An infective factor controlling sex compatibility in *Bacterium coli*. *J. gen. Microbiol.* **8**, 89.

CAVALLI-SFORZA, L. L. & JINKS, J. L. (1956). Studies on the genetic system of *Escherichia coli* K-12. *J. Genet.* **54**, 87.

CLOWES, R. C. & ROWLEY, D. (1954). Genetic investigation of inhibition by DL-norleucine of the K-12 strain of *Escherichia coli*. *J. gen. Microbiol.* **11**, 27.

DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J. Bact.* **60**, 17.

DE HAAN, P. G. (1954). Genetic recombination in *Escherichia coli* B. *Genetics*, **27**, 293.

FREDERICQ, P. (1948). Actions antibiotiques reciproques chez les Enterobacteriaceae. *Rev. belge Path.* **19**, Supplement IV, 32.

FREDERICQ, P. (1954*a*). Transduction génétique des propriétés colicinogènes chez *Escherichia coli* et *Shigella sonnei*. *C. R. Soc. Biol., Paris*, **148**, 399.

FREDERICQ, P. (1954*b*). Intervention du facteur de polarité, sexuelle F dans la transduction des propriétés colicinogènes chez *E. coli*. *C. R. Soc. Biol., Paris*, **148**, 746.

GRATIA, A. (1925). Sur un remarquable exemple d'antagonisme entre deux souches de Colibacille. *C. R. Soc. Biol., Paris*, **93**, 1040.

HAYES, W. (1952). Genetic recombination in *Bact. coli* K-12. Analysis of the stimulating effect of ultra violet light. *Nature, Lond.* **169**, 1017.

HAYES, W. (1953). Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. *J. gen. Microbiol.* **8**, 72.

HIROTA, Y. (1956). Artificial elimination of the F factor in *Bacterium coli*, K-12. *Nature, Lond.* **178**, 92.

JACOB, F. & WOLLMAN, E. L. (1954). Étapes de la recombinaison génétique chez *Escherichia coli* K-12. *C. R. Acad. Sci., Paris*, **240**, 2566.

LEDERBERG, J. (1950). Isolation and characterisation of biochemical mutants of bacteria. *Meth. med. Res.* **3**, 5.

LEDERBERG, J., CAVALLI, L. L. & LEDERBERG, E. M. (1952). Sex compatibility in *Escherichia coli*. *Genetics*, **37**, 720.

LEDERBERG, J. & LEDERBERG, E. M. (1956). *Mechanisms in differentiation and growth*, p. 101. Princeton University Press.

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Bisset, K. A. (1957). *J. gen. Microbiol.* **17**, 562–566

Some Observations upon the Mode of Sporulation and Relationships of Monosporous Actinomycetes

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SUMMARY: The modes of sporulation of *Micromonospora chalcea*, the parasitic *Actinomyces odontolyticus* and of stages in the primary mycelium of a strain of Streptomyces are compared. All produce spores singly on short side-branches, but the characters of the parasitic Actinomyces appear to be neotenic and degenerate, whereas those of the Streptomyces are primitive, and show signs of affiliations with a variety of different genera.

The classification of the actinomycetes given in *Bergey's Manual* (6th ed. 1948) is based upon that of Ørskov (1923), and although it is founded upon morphological considerations is notably defective in its information. This is especially true in regard to the parasitic microaerophiles, the true Actinomyces. The members of this genus possess a life-cycle of a comparable type to that of other actinomycetes (Morris, 1951; Prévot, 1953; Batty, 1957), but this has been overlooked by the earlier observers; *Bergey's Manual* (1948) simply states: 'No conidia produced'.

Although the mode of sporulation of Micromonospora is recorded in general terms: '... conidia produced singly at the end of special conidiophores' (*Bergey's Manual*), the details have never been investigated. The best illustration available is that of Morris (1952). Micromonospora is assigned to the same family as Streptomyces, but the differences between them are very considerable.

The mode of sporulation of the parasitic Actinomyces is so remarkable, that it has appeared curious to me that it should be found only in that genus and in Micromonospora; both of which are, in their respective ways, rather highly specialized. And repeated attempts have been made in this laboratory to discover comparable bacterial forms of a more generalized type. In particular, it would be of great interest, in view of past confusion concerning the nature of parasitic Actinomyces, to determine their free-living counterparts.

In this paper a strain of Streptomyces is described which reproduces itself indefinitely in the primary phase upon routine bacteriological media, and without forming an aerial mycelium produces spore-like bodies in a variety of forms, the morphology of which is compared with that of the spores of Micromonospora and Actinomyces. It is concluded that it is a relatively primitive form, whereas the parasitic *A. odontolyticus* is degenerate.

MATERIALS

Three strains of *Micromonospora chalcea* were examined; these were isolated in this laboratory by Mr L. A. Davey from cultivated soil.

Six strains of *Actinomyces odontolyticus* were examined; these were isolated in this laboratory, from human saliva and carious teeth by Miss I. Batty, by whom it is described (Batty, 1957). It closely resembles *A. bovis* in its life-cycle, but was chosen for this study because it is considerably easier to grow in artificial culture, after the initial difficulties of isolation have been overcome.

The strain of Streptomyces was isolated from soil in the course of a class exercise. It grows slowly on most standard media, at temperatures below 32° (approx.), and is not exacting in respect of pH value, but, of those media examined, does not produce an aerial mycelium except on malt or oatmeal agar. The colonies on agar are grey and waxy, resembling those of *Bacillus cereus*. They are not adherent to the medium. When the aerial mycelium is formed it is abundant, pink and adherent.

METHODS

The Micromonospora strains were grown upon a medium consisting of 1 % glucose, 0·05 % K_2HPO_4 and 0·05 % asparagine solidified with 1·5 % agar in tap water, at pH 6·8, at a temperature of 15° aerobically. The *Actinomyces odontolyticus* strains were grown on meat infusion agar with 10 % horse blood at pH 7·6, at 37°, in a closed jar filled with as many inoculated plates as it would contain. This induced moist, slightly microaerophilic conditions suitable to the growth of this species, which is rather more resistant to oxygen than are newly isolated strains of *A. bovis*. Streptomyces was grown on meat infusion agar for cytological examination of the primary mycelium.

Smears were stained by the method of Hale (1953) for cell walls.

RESULTS

The spores of *Micromonospora chalcea* were produced at all stages of growth of the colony, after the first 2 or 3 days. They could be seen simultaneously in the same culture, either upon long or short stalks or in clusters (Pl. 1, figs. 1–8). Thus the suggestion that these characters may have a taxonomic value (*Bergey's Manual*, 6th ed., 1948) is obviously without foundation. Yet another type of spore arrangement was frequently found, which controverts the definition of the genus, since these spores were not at the ends, but at some distance from the ends of the sporogenous filaments, with a projecting portion of the filament beyond (Pl. 1, figs, 9, 10). The immature spores were lanceolate (Pl. 1, figs. 1, 4) but they became spherical when mature. There was no sign, at any stage of growth, of anything resembling an aerial mycelium.

The spores of *Actinomyces odontolyticus* were produced in cultures from 1 or 2 weeks old, singly or in small groups on small pieces of fragmented mycelium. The stalk was always very short, and the spore retained the lanceolate shape to maturity (Pl. 1, figs. 11–15). The resemblance between the immature spores of the Micromonospora and the mature spores of the Actinomyces was very close (Pl. 1, figs. 1, 4 and 13–15). The arrangement of the larger groups of spores, on short side-branches, was also exceedingly similar in both genera (Pl. 1, figs. 7, 11, 15 and tracings). This resemblance extends to the process of formation of the spores, so far as it can be deduced from the appearances described (see Discussion).

The initial growth of the Streptomyces consisted, as in Actinomyces (Morris, 1951; Batty, 1957), of short, bacillary forms, but these were soon replaced by longer septate filaments, sparingly branched, upon which the spores were formed, in a variety of arrangements. Some of these bore a marked resemblance to the spores of Micromonospora (Pl. 2, figs. 16–20), but were less regularly spherical. Some were oblong, and the younger stages oval or lanceolate. There were also large spores which had the appearance of acorns, and were borne at the angle of a sharply bent filament (Pl. 2, figs. 16, 17 and 19). These had a distinct sign of segmentation at the base, representing the cup of the acorn. Other spores were borne at the tips of short filaments, and again they were not always spherical, but frequently oval or oblong (Pl. 2, figs. 21–24). This type of spore was also found to be segmented at the base in some instances (Pl. 2, figs. 23, 24). Occasionally a more complete segmented type of spore was seen, giving the appearance of a short chain (Pl. 2, figs. 25, 26).

In addition, a very characteristic appearance occurred quite commonly at all stages of culture. This took the form of pairs of oblong cells conjoined in a fashion exceedingly suggestive of conjugation (Pl. 2, figs 27–29), and especially reminiscent of the mode of formation of the ‘initial cells’ of the secondary mycelium in Actinomyces and Micromonospora described by Morris (1951, 1952), and in Streptomyces by Klieneberger-Nobel (1947) and Dickenson & Macdonald (1955). It is conceivable that the ‘acorn’ spores may be arrested initial cells.

These various appearances were also observed in the early stages of growth on media upon which the entire life-cycle was completed, with the formation of long chains of aerial spores.

DISCUSSION

The appearance of the immature and maturing spores of *Micromonospora chalcea* and *Actinomyces odontolyticus* suggests that these develop in the same way, as enlargements upon an existing filament, and not by outgrowth from the tip. This is clearly seen in both cases by the lanceolate form of the immature spore, which carries its major enlargement at some distance from the pointed tip, and which is as long or longer than the mature form in many cases. It is even more clearly seen in those spores of *M. chalcea* which are not produced at the end of the sporogenous filament but at some distance from it. Corresponding differences are seen in *A. odontolyticus* between spores with longer or blunter tips. The entire sporulation process in *A. odontolyticus* is suggestive of degeneracy and neoteny, as might reasonably be expected of a parasite. But the two processes resemble one another very closely, and may be presumed to indicate a relationship between these genera.

The arrest of the Streptomyces in the early stages of its life-cycle, presumably by reason of nutritional deficiency of substances present in malt and oatmeal, but absent in nutrient agar, offers an opportunity to study the curiously diverse modes of spore production in the pre-aerial mycelium. These spores

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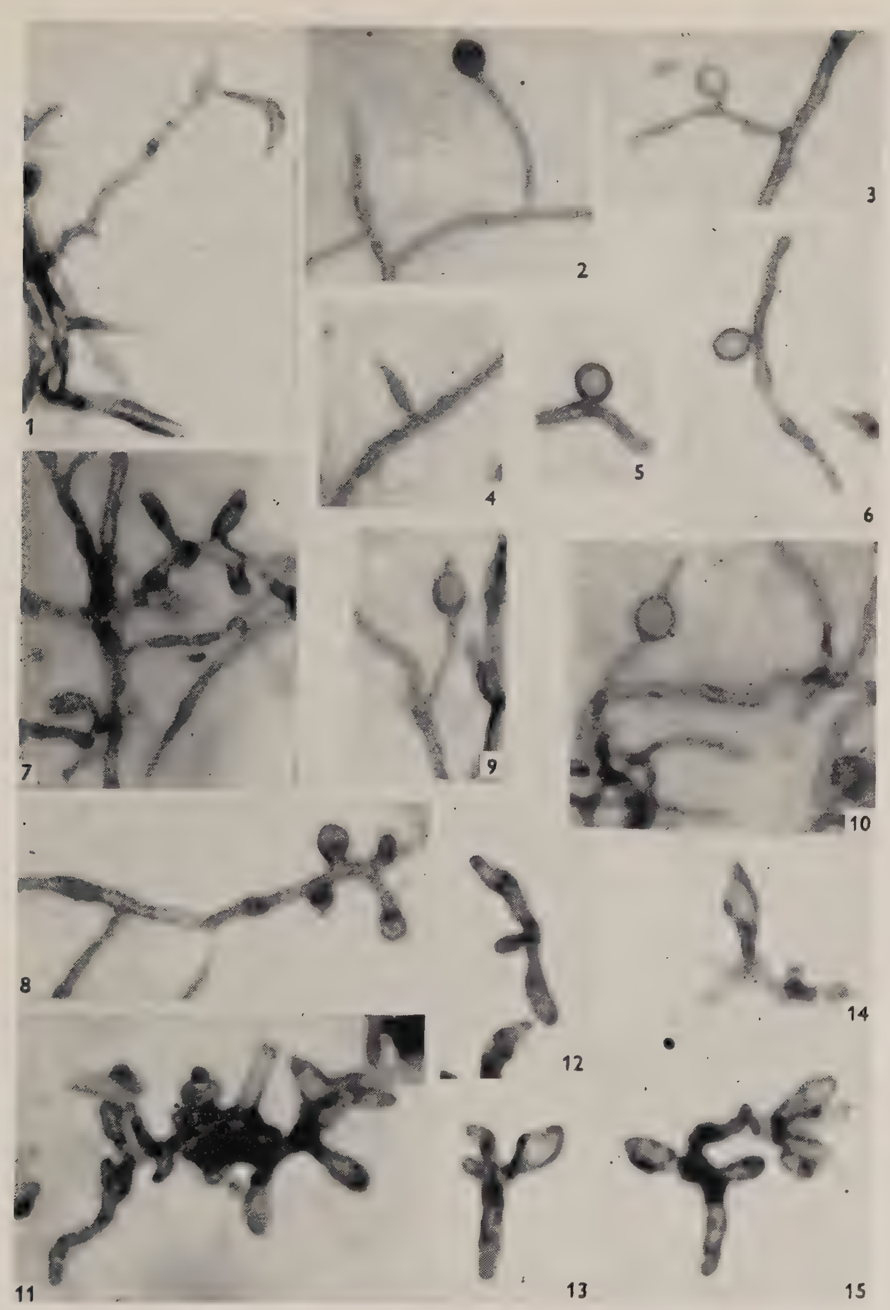
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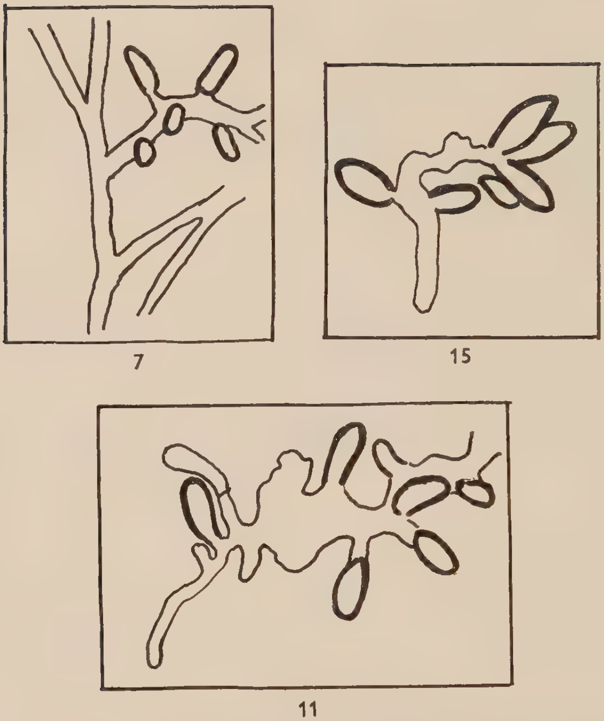
K. A. Bisset—Monosporous *Actinomycetes*. Plate 1

(*Facing p.* 564)



Tracings of Figs. 7, 11 and 15, showing groups of immature spores of *Micromonospora chalcea* compared with groups of maturing and mature spores of *Actinomyces odontolyticus*.

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Nitrogen Fixation by New Species of *Nocardia*

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SUMMARY: Two new species of the genus *Nocardia* (Trev.) have been isolated from the soil of chalk grassland. One of these, *Nocardia calcarea* n.sp., fixes atmospheric nitrogen in culture in amounts of 2–4·5 mg. N/g. glucose, sucrose or mannitol present in the medium. The second species, *N. cellulans* n.sp., decomposes cellulose in culture and fixes atmospheric nitrogen in culture using glucose, sucrose, mannitol and cellulose as carbon source; amounts of 12 mg. N fixed/g. cellulose decomposed were recorded. The properties of nitrogen fixation and cellulose decomposition have not previously been recorded for this genus. The two organisms are described.

The two species of *Nocardia* to be described were isolated in the course of an examination of the nitrogen-fixing and cellulose-decomposing bacteria in the soil beneath a chalk grassland plant community. As the ability to fix atmospheric nitrogen shown by both these organisms and the ability to decompose cellulose shown by one are attributes not previously recorded for the genus *Nocardia*, a determinative description of the two organisms (which are regarded as new species) is given. It is proposed to use the name *Nocardia cellulans* for the cellulose-decomposer and the name *N. calcarea* for the second organism, which as yet has been isolated only from chalk soil.

METHODS

Isolation of Nocardia calcarea. This organism appeared repeatedly in the course of isolations of species of *Azotobacter* in liquid enrichment media using chalk soil as inoculum. The 'nitrogen-free' medium had the composition: 3·0 g. K_2HPO_4; 1·0 g. NaCl; 0·01 g. $MgSO_4.7H_2O$; 0·01 g. $FeCl_3$; 1·0 g. $CaCO_3$; glass-distilled water, 1 l.; 1 p.p.m. $Na_2MoO_4.2H_2O$; 0·3% (w/v) sodium benzoate; pH 7·2. After three successive transfers at 20° in this medium the enriched cultures were plated on the same medium solidified with washed agar. The colonies of Nocardia were easily differentiated from those of Azotobacter by their pinkish colour. Final isolation was obtained as readily by using mannitol or glucose (1%, w/v) as the carbon supply. Isolation of the organism was also readily achieved by the 'spontaneous culture' method of Winogradsky (1949, p. 609) following the addition of sodium acetate to soil samples, also by a modification of his plate insemination method (Winogradsky, 1949, p. 626) in which washed agar was used to solidify the basal medium and butanol added as carbon source.

Isolation of Nocardia cellulans. A silica gel was used impregnated with basal medium (2·5 g. KNO_3; 1·0 g. K_2HPO_4; 0·1 g. $CaCl_2$; 0·3 g. $MgSO_4.7H_2O$; 0·1 g. NaCl; 0·01 g. $FeCl_3$; glass-distilled water, 1 l.; pH 7·0–7·2). Washed

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filter paper placed on the surface of the gel was seeded with minute soil crumbs. After incubating at 25° for 2–3 days, further plates were inoculated from the areas of decomposition around the crumbs. Mixed cultures were obtained which decomposed the filter paper rapidly and these were plated repeatedly on yeast-extract peptone agar. Between each of these platings the isolates were grown in tubes containing liquid basal medium with strips of washed filter paper. One of the most vigorous cultures so obtained proved to be a nocardia. Pure cultures of this organism grew well in liquid nitrogen-free media and on nitrogen-free agar media. Tests in the filter paper tubes described, with the omission of the KNO_3, showed that growth occurred with the simultaneous decomposition of the paper.

Nitrogen determinations. All estimations of nitrogen content were made by the semi-microKjeldahl method described by McKenzie & Wallace (1954) with mercuric sulphate as catalyst in digestion and added thiosulphate in distillation to break down catalyst complexes. Preliminary experiments showed that in liquid media the two organisms made best growth with mannitol as carbon source. Replicate samples (20 ml.; 0·5 %, w/v, mannitol; pH 7·0) of the nitrogen-free medium were inoculated with 1 ml. of saline suspension of bacteria; for subsequent use as controls some of the cultures were autoclaved just after inoculation. The cultures were incubated for 14 days at 25° and determinations of nitrogen content of grown cultures and of the autoclaved controls made. As for the present purposes it was only desired to demonstrate significant changes in nitrogen content, determinations were made on whole cultures which were acidified and evaporated to appropriate volumes on the water bath before digestion. In some experiments cultures and autoclaved controls were aerated continuously during incubation with a slow stream of sterile air freed from ammonia, others were incubated statically. Better growth was obtained in the aerated cultures.

Fixation of nitrogen by *Nocardia cellulans* with cellulose as carbon source was determined in samples (20 ml.; pH 7·0) of the basal medium without KNO_3 and with the addition of a known weight (about 0·4 g.) of washed filter paper. Aeration and the addition of yeast extract (Yeastrel or Marmite) at 1 p.p.m. produced greatly increased growth and cellulose decomposition. Residual cellulose was determined in about half the cultures before nitrogen estimations were made; the method of Fåhraeus (1947) was used with omission of the stage involving washing the residues with ammonia to remove mucilage.

NOCARDIA CALCAREA SP.NOV.

In 2-day culture, Gram positive and partially acid fast. The morphological cycle on nutrient agar is as follows. After 1–2 days at 25°, mainly short rods (1·5–2·0 × 1·0 μ.) together with unbranched aseptate filaments up to 10 μ. in length and occasional branched filaments. Some filaments show lemon-shaped swellings. After 4 days, mainly short rods showing snapping division typical of corynebacteria; abundant unbranched filaments (5·0–8·0 × 1·0 μ.) and a few branched filaments and cocci (1·0 μ.) also present. After 7 days mainly rods; cocci abundant but few filaments. At this stage many rods show the differentiation of a swollen spore-like structure; these are usually formed terminally or subterminally, one per rod. In the filaments they are often formed in chains. Examined in phase-contrast they are hyaline or slightly refractive. Colonies after 14 days consist of short rods, cocci and rods with swollen cells.

On glucose and mannitol agars growth is similar but very long branched filaments (10–25 μ.) are present after 2 days, often with terminal chains of swollen hyaline cells; these filaments usually fragment but a few persist. On yeast-extract peptone medium the cycle is shorter, most of the filaments having fragmented into rods and cocci after 3 days. On sucrose agar, filaments are rare and short rods and cocci are the predominant forms throughout. Occasional lemon-shaped cells are formed on all media.

Litmus milk. Heavy growth, turned alkaline, no peptonization.

Gelatin stab. Beaded growth at top of stab, no liquefaction.

Starch agar. Slight growth, no hydrolysis detectable.

Nitrate peptone broth. Heavy uniform growth, nitrite produced.

Carbohydrate metabolism. In basal salts medium with $NH_4H_2PO_4$ and bromcresol purple, heavy growth with glucose and sucrose; acid produced. Growth good with maltose, poor with lactose.

Paraffin utilization. Growth heavy in basal salts medium with $NH_4H_2PO_4$ and flakes of paraffin wax.

Colonies on agar media. Circular, raised, soft, without aerial mycelium, pink or cream with distinct pink tinge when small, never brightly coloured.

NOCARDIA CELLULANS SP.NOV.

In 2-day culture, Gram positive and partially acid fast. The morphological cycle on nutrient agar is as follows. After 2 days' incubation at 25° the growth consists of branching aseptate filaments, 30–40 μ. in length, often with swellings at intervals; shorter filaments are present but few are less than 7·0 μ. in length. About the 4th day fragmentation commences and the number of short rods (1·5–2·0 × 1·0 μ.) increases rapidly. Branched filaments (up to 10 μ. in length) are present until the 10th day or later. Hyaline spore-like structures are produced from the 7th day as slight terminal swellings on the filaments. After 28 days the colonies consist of very short rods, cocci and spore-like cells.

The morphological cycle on yeast-extract peptone agar is very similar. On glucose agar the filaments fragment less rapidly and are occasionally found after 28 days. Numerous Y-forms are found in older cultures. On sucrose agar the filaments seldom exceed 14 μ. in length. In cellulose tubes after 6 days there are long (20–30 μ.) branched and unbranched filaments, many with terminal swellings. Fragmentation is rapid and short rods and cocci predominate during the stage of active cellulose decomposition. Old cultures are composed almost entirely of cocci.

Litmus milk. Acid and curd are produced.

Gelatin stab. Beaded growth at top of stab; no liquefaction.

Starch agar. Starch is not hydrolysed.

Nitrate peptone broth. Heavy, uniform growth; nitrite produced.

Carbohydrate metabolism. In basal salts medium with $NH_4H_2PO_4$ and bromcresol purple, heavy growth with glucose, sucrose and maltose; acid produced.

Paraffin utilization. Growth is heavy in basal salts medium with $NH_4H_2PO_4$ and flakes of paraffin wax if trace of yeast-extract added; no growth without yeast-extract.

Colonies on agar media. Entire, raised, soft, without aerial mycelium, cream in colour on most media but characteristically bright yellow on yeast-extract peptone agar.

NITROGEN FIXATION

Typical values for nitrogen fixation under the different conditions used are shown in Table 1. There were differences in the degree of fixation and in the utilization of the mannitol between replicate cultures; the same variation was obtained with *Azotobacter* sp. under the same conditions. Attempts to estimate residual mannitol in the cultures by the method of Cameron, Ross & Percival (1948) were abandoned as (due to the apparent production of reducing substances in the medium as by-products) consistency could not be obtained for replicate samples of the same culture. Not all the mannitol available had been used.

Table 1. *Typical amounts of nitrogen fixed under different conditions by certain* Nocardia *spp.*

For comparison, values are given for nitrogen fixation by *Azotobacter* sp. isolated from chalk soil.

Organism	Conditions	Nitrogen fixed (mg. bacterial-N/g. available substrate)
Nocardia calcarea	Mannitol 0·5 %, still cultures	2·0–4·0
	Mannitol 0·5 %, aerated cultures	3·0–4·5
	Mannitol 0·5 %, with yeast, aerated	4·0
	Glucose 1·0 %, with yeast, aerated	2·0
	Sucrose 1·0 %, with yeast, aerated	2·5
N. cellulans	Mannitol 1·0 %, with yeast, aerated	3·0–4·0
Azotobacter sp.	Mannitol 1·0 %, aerated cultures	7·0–9·0
		(mg. bacterial-N/g. cellulose decomposed)
N. cellulans	Cellulose, aerated cultures	5·0–9·0
	Cellulose, with yeast, aerated	5·0–12·0

Quicker initial growth and the higher values of fixation were obtained by adding yeast-extract (Yeastrel or Marmite) to the medium in a concentration of 1 p.p.m. This beneficial effect of added yeast-extract was especially marked with *Nocardia cellulans.* The constituent of the extract responsible has not been identified. Neither organism seems to require thiamine or biotin and adding these vitamins to the nitrogen-deficient medium produced no stimulation of growth nor was fixation enhanced. Addition of some of the amino acids which are most abundant in yeast-extracts (Special Report, 1956) and of other nitrogenous compounds at concentrations of 1 p.p.m. and 10 p.p.m. of fixed nitrogen appeared to suppress fixation. The compounds tested were glutamic acid, leucine, aspartic acid, β-alanine, $NH_4H_2PO_4$. At 1 p.p.m. of N all gave fair growth but with doubtful fixation; at 10 p.p.m. there was heavy growth with all except β-alanine and no fixation.

Long-period serial transfer and storage on nitrogen-deficient medium results, for both organisms, in a gradual loss of ability to fix nitrogen; this ability is restored by transfer to sterile soil. Serial transfer on media such as yeast-extract peptone results in a very rapid loss of fixing ability and, with *Nocardia cellulans*, loss of ability to decompose cellulose; passage through sterile soil may or may not restore these properties. Both organisms survive and retain their properties over a period of years in damp or air-dry sterile soil.

DISCUSSION

In view of the wide variety of bacteria which have now been shown to fix atmospheric nitrogen, it is interesting to find members of the Actinomycetaceae that are able to do so. Jensen (1931) stated that no organism belonging to Group II of his classification (the non-proteolytic, non-diastatic group of *Norcadia* of *Bergey's Manual*, 1948) could decompose cellulose; *Nocardia cellulans* would appear to belong to this group. Many species of *Streptomyces* can decompose cellulose and Jensen (1940) isolated three species of *Corynebacterium* with this property; it is therefore not surprising to find a cellulose-decomposing member in the related genus *Nocardia*. The ability to use cellulose directly as the carbon source for nitrogen fixation is novel, but is consistent with the findings of Jensen & Swaby (1941) and of Jensen (1941) that *Azotobacter* spp. and *Clostridium butyricum* can utilize (in nitrogen fixation) the metabolic products formed during cellulose decomposition by a variety of organisms. The present authors (unpublished work) have found that the products of cellulose decomposition by *Nocardia cellulans* can thus feed azotobacters during nitrogen fixation.

Nocardia cellulans and *N. calcarea* may be placed in the non-proteolytic, non-diastatic group of nocardias. Within the group, *N. cellulans* resembles *N. polychromogenes*, *N. opaca* and *N. erythropolis* in forming extensive initial mycelium and also forming soft colonies without visible aerial mycelium. It differs from these species in other colony characters, in morphology on nutrient agar, in growth characters and reaction in litmus milk and in producing acid from glucose, sucrose and maltose. Within the group, *N. calcarea* has affinities with *N. salmonicolor*, in that the initial mycelium is usually limited and soon breaks up into rods and cocci, but it differs from this species in colony characters, in morphology on nutrient agar, in growth characters on gelatin and in litmus milk and in producing acid from glucose and sucrose.

Active cultures of *Nocardia calcarea* and *N. cellulans* have been deposited in the type-culture collection at the National Collection of Industrial Bacteria, Chemical Research Laboratory, Teddington, Middlesex.

REFERENCES

Bergey's Manual of Determinative Bacteriology (1948). Sixth ed. Eds. Breed, R. S., Murray, E. G. D. and Hitchens, A. P. London: Baillière, Tindall and Cox.

Cameron, M. C., Ross, A. G. & Percival, E. G. V. (1948). Methods for the routine estimation of mannitol, alginic acid and combined fucose in seaweeds. *J. Soc. chem. Ind.* **67**, 161.

Fåhraeus, G. (1947). Studies in the cellulose decomposition by Cytophaga. *Symb. bot. upsaliens.* **9**, 2.

Jensen, H. L. (1931). Contributions to our knowledge of Actinomycetales. II. *Proc. Linn. Soc. N.S.W.* **56**, 345.

Jensen, H. L. (1940). Nitrogen fixation and cellulose decomposition by soil microorganisms. I. *Proc. Linn. Soc. N.S.W.* **65**, 543.

Jensen, H. L. (1941). Nitrogen fixation and cellulose decomposition by soil microorganisms. III. *Proc. Linn. Soc. N.S.W.* **66**, 239.

Jensen, H. L. & Swaby, R. J. (1941). Nitrogen fixation and cellulose decomposition by soil micro-organisms. II. *Proc. Linn. Soc. N.S.W.* **66**, 89.

McKenzie, H. A. & Wallace, H. S. (1954). The Kjeldahl determination of nitrogen: a critical study of digestion conditions—temperature, catalyst and oxidising agent. *Aust. J. Chem.* **7**, 55.

Special Report (1956). *Constituents of Bacteriological Culture Media.* Special Report by the *Soc. gen. Microbiol.* Cambridge University Press.

Winogradsky, S. (1949). *Microbiologie du Sol.* Paris: Masson et Cie.

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WINDER, F. G. & DENNENY, J. M. (1957). *J. gen. Microbiol.* 17, 573–585

The Metabolism of Inorganic Polyphosphate in Mycobacteria

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SUMMARY: Cell-free extracts of *Mycobacterium smegmatis* contain an inorganic polyphosphatase and a 'polyphosphate-AMP-phosphotransferase'. The latter enzyme, which synthesizes adenosine triphosphate from adenosine-5-monophosphate and inorganic polyphosphate, has a pH optimum of about 6·3, and is stimulated by Mg. It does not appear to be attached to cell particles, and a slight purification may be effected by ammonium sulphate precipitation.

With a tracer technique it was shown that cellular inorganic polyphosphate is probably not the sole precursor either of lipid phosphorus or of nucleic acid phosphorus in *M. smegmatis*. The rapid accumulation of insoluble polyphosphate caused in *M. smegmatis* and *M. phlei* but not *M. tuberculosis*, by a number of organic substances, particularly alcohols, was studied. This accumulation takes place even after inoculation into a fresh nitrogenous medium, when polyphosphate normally decreases.

In a previous paper (Winder & Denneny, 1956) we reported studies on the phosphorus-containing components of some mycobacteria. All the organisms studied contained inorganic polyphosphates. Although these substances occur in many lower plants, and in some animals, their metabolism has been the subject of comparatively little study. Polyphosphate metabolism in mycobacteria has been investigated by Ruska, Bringmann, Neckel & Schuster (1952), Meissner & Kropp (1953), Meissner & Diller (1953) and Meissner & Lemke (1955). The studies reported here were directed towards the elucidation of the following questions in the case of the mycobacteria. Are inorganic polyphosphates the direct source of other high-energy phosphorus compounds? Have they a close metabolic connexion with any other phosphorus fractions? How is their metabolism controlled in the cell?

METHODS

Organisms used. The organisms used and the conditions for growth have already been described (Winder & Denneny, 1956).

Polyphosphate accumulation; short-term experiments. These experiments were similar to the previously described short-term growth experiments but with appropriately changed media; and the samples were treated and the phosphorus compounds extracted and determined as previously described (Winder & Denneny, 1956).

Experiments with radioactive phosphorus. Four-day cultures of *Mycobacterium smegmatis* were washed with Proskauer & Beck medium (P & B) (American Trudeau Society, 1950) from which the nitrogen source (asparagine) was omitted, suspended by grinding in the same medium in a ball-mill, and brought to a standard turbidity (equivalent to *c.* 4 mg. dry wt./ml.). Forty ml. of this suspension were pipetted into each of two 150 ml. flasks containing 20 ml. of nitrogen-free P & B medium, 2 ml. 65% (w/v) tetrahydrofurfuryl alcohol (THFA) in water and 2 ml. of 0·5% (w/v) Tween 80. In one flask the phosphorus contained 0·5 mc of ^{32}P. The flasks were shaken in a water-bath at 37° for 2 hr. while polyphosphate accumulation took place. The contents were then centrifuged, and the organisms washed twice by stirring in nitrogen-free P & B medium and recentrifuging, suspended in 60 ml. P & B medium and transferred to 150 ml. flasks containing 2 ml. 0·5% (w/v) Tween 80. To the flasks containing the unlabelled organisms 0·5 mc of ^{32}P were added. Samples were withdrawn at once and the flasks shaken at 37° in the water bath. Further samples were withdrawn when required. The samples were washed and fractionated as described previously. Phosphorus determinations and ^{32}P counts were done on all the fractions, and on the medium. The specific activity of the inorganic polyphosphate was determined after precipitation with barium chloride at pH 4·5. In the acid-soluble fraction, the orthophosphate and labile phosphates were precipitated with magnesia mixture after hydrolysis for 10 min. with N-H_2SO_4, and the specific activity of this precipitated phosphate was determined. From the figures obtained the concentrations and the specific activities of the lipid phosphorus, 'phosphoprotein', 'insoluble' polyphosphate, labile and stable acid-soluble phosphate, deoxypentosenucleic acid and pentosenucleic acid were calculated. Both nucleic acids contained, however, some uncharacterized phosphate.

Counting of radioactive samples. Samples (0·3 ml.) of the solutions (all in 5% trichloroacetic acid, TCA) were pipetted on to nickel planchettes and counted immediately under an end-window counter. Duplicate samples of each solution were counted, 10,000 counts being taken on each sample. Where duplicates did not agree to within 2%, further samples were counted and the mean taken.

Preparation of cell-free extracts. Mycobacterium smegmatis from 4-day cultures was vigorously ground for 10–15 min. by hand in a chilled mortar with about the same weight of acid-washed alumina and enough cold 0·1 M-phosphate buffer (pH 7·2) to make a slurry. (Use of insufficient buffer during the grinding may result in inactivation of enzyme.) The slurry was diluted with the buffer, chilled and centrifuged for 3 × 10 min. periods at about 3000 *g* to remove alumina, organisms and large cell-fragments. The extract was dialysed overnight at about 4° against distilled water, and recentrifuged.

Estimation of polyphosphate-AMP-phosphotransferase

Method (i). This method relied on the presence in the extract of glycerol-kinase, which caused the transfer of phosphate from adenosine triphosphate (ATP) or adenosine diphosphate (ADP) to glycerol, a reaction which went nearly to completion. One ml. of 0·5% (w/v) $MgSO_4 . 7H_2O$, 1 ml. of 2% (w/v) glycerol in water, and 1 ml. of 0·5 M-succinate buffer (pH 6·3), were pipetted into a test tube and 1 ml. of a 0·2% (w/v) solution of adenosine-5-monophosphate (AMP), and 1 ml. of sodium pholyphosphate solution were added. Three ml. of cell-extract was added, the solutions mixed, and 2 ml. samples pipetted into 2 ml. volumes of 10% trichloroacetic acid (TCA). Control tubes lacking polyphosphate and nucleotide were also set up. The tubes were stoppered, incubated at 37° for 3–5 hr. and further samples were taken. The sample tubes were capped, heated for 10 min. at 90° (to extract the polyphosphate from the precipitated protein), centrifuged, and the supernatant fluid used for the determination of the sum of inorganic and acid-labile phosphate. The decrease in this figure during incubation was a measure of the amount of stable phosphate ester (glycerophosphoric acid) formed and hence, for the reason mentioned above, a measure of the formation of ATP and ADP.

Method (ii). This relied on the direct determination of the nucleotides. The incubation mixture was the same as in Method (i), but without glycerol. Control tubes were incubated without AMP and polyphosphate. After 3 hr. 6 ml. 10% (w/v) TCA were added to each tube. The tubes were then left in the cold for 1 hr., extracted twice with 20 ml. lots of ether (to decrease the TCA content) and neutralized. The solutions were put on Dowex 1 columns and the nucleotides eluted according to the method of Cohn & Carter (1950). The eluates were collected in 10 ml. lots and their absorption read at 260 mμ.

Chemicals. The inorganic polyphosphate used was sodium metaphosphate (Hopkin & Williams). A 2% (w/v) suspension in 1% (w/v) K_2SO_4 was adjusted to pH 7·4, kept overnight, its pH value readjusted to the required value, the suspension centrifuged, and the supernatant fluid diluted 1:1 with water.

Adenosine-5-monophosphoric acid, adenosine-3-monophosphoric acid (A-3-MP) and other nucleoside derivatives were obtained from Schwarz Laboratories, New York 17, except ADP and ATP which were obtained as the barium salts from L. Light and Co. Colnbrook, Bucks.

RESULTS

Conditions for abnormal polyphosphate accumulation

When material from a 4-day culture of *Mycobacterium smegmatis* was incubated in nitrogen-free P & B medium, little further accumulation of insoluble polyphosphate took place. When, however, 2% tetrahydrofurfuryl alcohol (THFA) was included in the medium a rapid accumulation of insoluble polyphosphate was observed (Table 1) as previously reported (Winder & Denneny, 1954). There was no substantial change in any of the other fractions, except on occasion an increase in the acid-soluble labile phosphate. This was not found in the experiment illustrated in Table 1. It was found that when *M. smegmatis* was grown in the presence of THFA the organisms contained a greatly increased concentration of polyphosphate, both soluble and insoluble (Table 2). There was a slight increase in most other phosphorus fractions and a decrease in the deoxyribonucleic acid (DNA) content.

In order to explain the accumulation of polyphosphate, it was first postulated that THFA inhibited its utilization. On incubating *Mycobacterium smegmatis*

Table 1. *Effect of tetrahydrofurfuryl alcohol (THFA) on changes in phosphorus fractions of* Mycobacterium smegmatis *shaken at 37° as a heavy suspension in nitrogen-free P & B medium containing various concentrations of phosphate*

Concentration of THFA (%, w/v) ...		0			2								
Concentration of KH_2PO_4 (%, w/v) ...		0·25			0·25			0·05			0·01		
Period of incubation (hr.)	0	1	3	5	1	3	5	1	3	5	1	3	5
	μg. P/mg. cell nitrogen												
Lipid	11·7	12·4	13·0	13·1	12·8	13·1	14·1	—	—	—	—	—	—
Protein P	6·7	—	8·2	8·5	7·7	8·3	9·2	—	—	—	—	—	—
Acid-soluble P													
(i) Orthophosphate	12·5	11·6	12·7	13·9	11·6	12·5	13·1	8·7	11·0	10·0	8·7	9·2	9·0
(ii) Acid-labile P	7·7	8·5	8·5	7·6	8·1	8·5	8·0	7·5	7·0	9·4	6·6	7·1	7·1
(iii) Stable P	22·5	17·9	17·4	16·9	19·5	18·5	18·5	—	—	—	—	—	—
Nucleic acid P	68	66	71	71	65	66	68	—	—	—	—	—	—
Insoluble polyphosphate	130	124	130	130	132	168	191	142	181	228	143	167	153

in nitrogen-free P & B medium containing ^{32}P it was found, however, that THFA strongly stimulated the incorporation of ^{32}P into polyphosphate. Another possibility considered was that THFA damaged the organisms in such a way as to permit the free entry of external phosphate, and that the organisms responded by forming polyphosphate to decrease the amount of the orthophosphate. This theory was not supported by studies on the effect of changing the concentration of phosphate in the medium (Table 1). Accumulation was still observed with 0·01 % (w/v) KH_2PO_4, although the organisms contained a higher orthophosphate concentration. Further, with low external phosphate and THFA, the concentration of cellular orthophosphate dropped while polyphosphate accumulation took place (Table 1). The results, therefore, force the conclusion that THFA stimulated polyphosphate synthesis either by direct stimulation of the enzymes involved, by inactivation of some controlling mechanism, or by causing some structural disorganization so that in its presence increased amounts of phosphate reach the polyphosphate-forming regions. There appears to be no serious damage to the organisms, however, since Table 2 shows accumulation of polyphosphate in the presence of THFA in organisms which were still growing vigorously. It was found too that the number of viable organisms was almost unaffected by a 5 hr. incubation with 5 % (w/v) THFA.

Table 2. *Effect of tetrahydrofurfuryl alcohol (THFA) in growth medium (P & B) on composition of* Mycobacterium smegmatis *harvested after* 6 *days of incubation*

THFA concentration (%, w/v) ...	1	0·7	0·3	0·13	0·07	0
	μg. P/mg. cell nitrogen					
Lipid-P	16·3	15·2	16·4	13·7	14·1	15·0
Acid-soluble P						
(i) Orthophosphate	16·2	12·9	11·5	12·0	10·6	13·0
(ii) Acid-labile P	23·1	12·1	10·1	7·8	5·2	5·7
(iii) Stable P	25·6	23·3	21·8	20·6	18·5	19·5
PNA-P	59·1	61·3	61·5	58·4	57·2	53·8
DNA-P	42·6	42·6	40·6	38·8	53·5	56·3
Insoluble polyphosphate	297	242	189	149	130	125

In the absence of THFA the addition of nitrogen to the incubation medium resulted in a decreased concentration of polyphosphate in *Mycobacterium smegmatis*, but when THFA was present the addition of nitrogen did not lead to a decreased formation of polyphosphate, although the utilization of phosphate for other purposes was increased (Table 3). The significance of this will be discussed later. Table 3 also shows that in the presence of nitrogen THFA had further effects and in particular caused an inhibition of DNA synthesis.

THFA caused rapid polyphosphate accumulation only over a narrow range of concentrations; at 0·5 % (w/v) the effect was slight and at 5 % (w/v) it was past its optimum, which was at about 2 % (w/v). Over longer periods, however, concentrations of THFA even below 0·1% (w/v) caused some polyphosphate accumulation (Table 2).

A number of substances other than THFA were tested for their effect on polyphosphate accumulation (Table 4). The list is too brief to show definitely what chemical or physical properties are associated with stimulation of polyphosphate accumulation; the active substances are all lipid-soluble.

Table 3. *Effect of tetrahydrofurfuryl alcohol (THFA) on changes in phosphorus fractions of* Mycobacterium smegmatis *shaken at 37° as a heavy suspension in P & B medium with and without a nitrogen source*

Concentration of asparagine (%, w/v) ...		0		0·5			
Concentration of THFA (%, w/v) ...		2		0		2	
Period of incubation (hr.) ...	0	5	11	5	11	5	11
	µg. P/10 ml. suspension						
Lipid P	30·9	29·8	39·3	30·0	39·5	29·3	34·7
Protein P	13·8	17·2	17·9	17·5	23·1	17·0	25·2
Acid-soluble P (excluding orthophosphate)	46·6	54·1	65·9	47·8	65·8	48·6	63·2
PNA-P	99·9	94·2	87·3	105	140	99·6	141
DNA-P	47·1	49·2	50·0	53·0	69·8	49·4	54·2
Insoluble polyphosphate	339	408	597	278	232	422	592
	mg. N/10 ml. suspension						
Total nitrogen	2·60	2·66	2·63	2·74	3·75	2·67	3·51

Table 4. *Comparison of a number of chemicals with tetrahydrofurfuryl alcohol (THFA) as stimulators of polyphosphate accumulation in* Mycobacterium smegmatis *shaken with them for 3 hr. at 37° in nitrogen-free P & B medium*

Chemical	Concentration (%, w/v)	Increase in polyphosphate as a % of that caused by 2·5% (w/v) THFA
Ethanol	2·0	25
	1·6	32
Tetrahydrofuran	2·0	32
	1·6	89
Butanol	1·0	91
Acetaldehyde	2·0	Decrease
	0·3	63
Propylene glycol	2·0	0
Butyrate	1·0	Decrease
Caproate	0·5	Decrease
	0·1	Decrease
Salicylate	0·6	Decrease
Hydroquinone	0·2	Decrease
Ferric chloride	1·0	Decrease
Chloromycetin	0·0005	0
Anilinoaposafranine	0·004	Decrease

At concentrations of up to 3% (w/v), THFA had no immediate effect on the respiration of *Mycobacterium smegmatis* in nitrogen-free P & B medium, but after 5 hr. of incubation with 0·5–2% (w/v) THFA, respiration decreased by *c.* 10%. When THFA was supplied as the sole respiratory substrate it was apparently metabolized, as the respiration rate was raised over the endogenous level and remained above that level for 5 hr.

The omission of either K or Mg from the incubation medium decreased the accumulation of polyphosphate in the presence of THFA. No attempt was made to eliminate all traces of these metals, so it cannot be stated whether there is an absolute requirement for either of these elements for polyphosphate formation.

2,4-Dinitrophenol (2 mM) caused complete inhibition of polyphosphate accumulation, with only 30% inhibition of respiration. At a concentration of 1 mM it caused 70% inhibition of accumulation with little or no effect on respiration. Sodium azide (9 mM) caused 45% inhibition of accumulation with 10% inhibition of respiration, and at 3 mM it caused 30% inhibition with no effect on respiration.

This stimulation of polyphosphate formation by THFA was not confined to *Mycobacterium smegmatis*; it was also shown by *M. phlei*, though not as strongly. *M. tuberculosis* H 37 Ra and R 1 Ra did not show this effect; virulent strains of *M. tuberculosis* were not investigated.

Isotope studies on metabolic function of polyphosphate

The labelling of the different phosphorus fractions was followed by adding labelled orthophosphate to growing cultures of *Mycobacterium smegmatis.* Insoluble polyphosphate was labelled less rapidly than cellular inorganic phosphate, and more rapidly than the various fractions insoluble in cold TCA. To determine whether insoluble polyphosphate was the exclusive precursor of any other phosphorus fraction, the experiment described under Methods was carried out. This consisted of two simultaneous and identical growth experiments, one starting with labelled external orthophosphate, and the other starting with most of the ^{32}P in the cellular polyphosphate.

If the system of an exclusive precursor A transferring phosphorus to a product B, which may in turn transfer it to other products (including back to A), is considered, the following relationship holds for any short period:

$$a = \frac{\Delta {}^{32}P_B - \Delta P_B \times \text{act}_B}{\text{act}_A - \text{act}_B}.$$

(where a is the phosphorus gained by B from A during the period, $\Delta {}^{32}P_B$ is the net increase in ^{32}P in B during the period, ΔP_B is the net increase in total phosphorus in B during the period, act_A and act_B are the mean specific activities of A and B during the period). Hence, to determine whether any fraction, A, is the exclusive precursor of any other fraction, B, the value of a can be calculated for any period in the experiment, both for the cells in the labelled phosphate medium and those with labelled polyphosphate. For each period the value of *a* obtained in the two cases should be the same, if the assumed precursor-product relationship is correct.

The results of the experiments (some of which are given in Table 5) were studied in this way for possible precursor-product relationships. Polyphosphate was not the exclusive precursor of either of the nucleic acid fractions (though the presence of some uncharacterized phosphate left some doubt), nor of phospholipid. Likewise, the origin of polyphosphate could not be identified with any of the other fractions studied. The results suggest that the precursor of polyphosphate is closely related to the external phosphate, and hence is probably one of the acid-soluble fractions. Further experiments

Table 5. *The distribution of* ^{32}P *from labelled external phosphate and cellular polyphosphate during the growth of* Mycobacterium smegmatis *in P & B medium*

Period of incubation (hr.)	P content of fraction (μg. P/10 ml. suspension)	Specific activity (counts/min./μg. P)		True PNA (μg. P/10 ml.)	True DNA (μg. P/10 ml.)
		Flask A	Flask B		
		External orthophosphate			
0·0		0·0	970		
		Insoluble polyphosphate			
0·0	760	72·5	0·0		
1·4	774	88·5	24·5		
3·0	718	88·8	48·5		
6·0	584	67·5	91·0		
10·2	452	55·0	171·6		
		Phospholipid			
0·0	21·0	11·4	0·0		
1·4	24·7	25·3	12·0		
3·0	29·6	38·3	51·3		
6·0	33·1	51·7	123·0		
10·2	35·2	41·7	218·0		
		PNA + unknown P			
0·0	365	18·9	0·0	262	
1·4	370	28·1	9·2	266	
3·0	371	34·3	34·2	270	
6·0	386	68·7	85·5	273	
10·2	430	58·7	161·5	326	
		DNA + unknown P			
0·0	144	5·2	0·0		130
1·4	148	22·9	9·6		133
3·0	153	33·0	28·3		133
6·0	173	—	72·4		—
10·2	210	65·0	182·8		182
		Stable acid-soluble P			
0·0	120·3	194	168		
1·4	100·7	97·3	205		
3·0	98·5	81·6	368		
6·0	—	—	—		
10·2	78·0	66·5	461		

along the same lines, with a full analysis of the acid-soluble components, are required to obtain a full understanding of the metabolic relationships of polyphosphate.

Metabolism of polyphosphate by cell-free extracts of Mycobacterium smegmatis

Cell-free extracts of *Mycobacterium smegmatis* contain an inorganic polyphosphatase stimulated by magnesium ions, with an optimum activity above pH 7. This enzyme was not investigated further. It was also found that the extracts contained an enzyme which appeared to be capable of using inorganic polyphosphate for the synthesis of ATP from ADP (Winder & Denneny, 1955). The presence of this enzyme was first established by the indirect method of showing an increased synthesis of glycerophosphoric acid from glycerol and ATP in the presence of inorganic polyphosphate (see Methods). Since, under the conditions of these experiments neither polyphosphate alone, nor polyphosphate + AMP, was able to cause an appreciable phosphorylation of glycerol it appeared that polyphosphate served to regenerate ATP from ADP. However, analysis on Dowex 1 ion exchange resin (see Methods) of the nucleotides formed on incubation of the extracts with polyphosphate and ADP showed that no ATP was formed. Synthesis of ADP from AMP and polyphosphate was, however, found to occur when the system was buffered at pH 6·3. Further work explained the earlier negative results obtained with AMP. The enzyme involved had a pH optimum between 6·0 and 6·5, and showed little activity at pH 7; the earlier work had been done at pH 7·4, but the system was weakly buffered so that when ATP or ADP was incubated the pH value was brought to 6·5 by phosphatase action. When AMP was added instead, the decrease in pH value was small and consequently the transphosphorylating enzyme was inactive. When the system was buffered at pH 6·3, the extract was able to phosphorylate glycerol, using AMP and polyphosphate. Hence, the enzyme is best described as a polyphosphate-AMP-phosphotransferase. Centrifugation at 25,000 *g* for 1 hr. left all the activity in the supernatant fluid. Hence, the enzyme appeared to be in solution in the extracts, though attachment to small particles was not excluded. Only preliminary work has been done on the purification of this enzyme. It was inactivated by precipitation with acetone. Fractionation with ammonium sulphate was more successful, most of the activity being precipitated between 1/4 and 1/2 saturation. A partial purification was carried out as follows: the undialysed extract was saturated with ammonium sulphate, left for 1 hr. at 4° and centrifuged. The precipitate was dissolved in 0·1 M-phosphate buffer (pH 7·2) ammonium sulphate added to half saturation, the suspension left for 1 hr. at 4° and centrifuged. The precipitate was dissolved in phosphate buffer, and the solution again centrifuged. The clear solution contained 50 % of the initial phosphotransferase, with considerably decreased polyphosphatase and other phosphatase activities, but it still contained the glycerolkinase.

A complete characterization of the enzyme must await further purification, on account of the polyphosphatases still present in the extracts. As already mentioned the optimum pH lies in the range of 6·0–6·5. The enzyme is stimulated by Mg at a concentration of 0·0003 M. It was active in the absence of added Mg, but this may have been due to traces of Mg in the extract.

The polyphosphate used contained a range of products from orthophosphate to high molecular weight polymers. When it was freed from the dialysable material it still served as a substrate for the polyphosphate-AMP-phosphotransferase. Short-chain polymers were not tested, except pyrophosphate, which did not serve as a substrate.

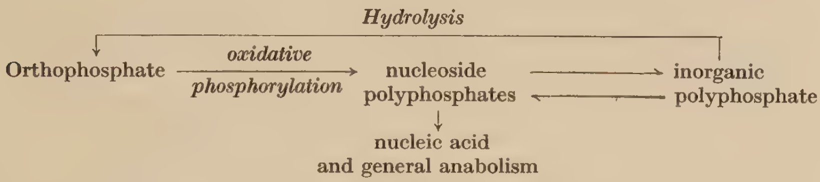
It appears that there are a number of enzymes for polyphosphate utilization present in *Mycobacterium smegmatis*. Some nucleosides (guanosine and uridine) were phosphorylated to a slight extent by the extracts in the presence of polyphosphate, while A-3-MP was also phosphorylated. These effects, however, were slight and require further investigation. The extracts appeared to lack a myokinase type of enzyme, as formation of ADP from AMP does not result in any increase in ATP. An enzyme was present for the direct phosphorylation of glycerol from ADP. The presence of a myokinase has been shown (Oliver & Peel, 1956) in *M. phlei* extracts, however.

DISCUSSION

The available evidence suggests that inorganic polyphosphates are synthesized in the cell from nucleoside polyphosphates (Yoshida & Yamataka, 1953; Kornberg, Kornberg & Simms, 1956; Lieberman & Eto, 1956). They are utilized either by hydrolysis to orthophosphate (polyphosphatases are present in a wide range of organisms) or by reversal of the synthetic reactions (Hoffmann-Ostenhof *et al.* 1954; Winder & Denneny, 1955). The results in this paper show that mycobacteria contain enzymes for the utilization of inorganic polyphosphates by both of these pathways. The value of inorganic polyphosphates in mycobacteria, and indeed in other organisms, is not clear, however. The polyphosphate in a 'resting' mycobacterial cell could provide enough phosphorus for another cell generation, but as a source of energy it is comparatively insignificant. The suggestion has been made that these polyphosphates have some special function connected with nucleic acid or protein synthesis, but no reliable evidence for this has been forthcoming. Our results with labelled phosphorus suggest that inorganic polyphosphates are not the sole precursors either of nucleic acid or lipid phosphorus, and are in keeping with the idea that they are utilized via the pool of acid-soluble phosphorus compounds. However, the results are not conclusive, nor was the work brought far enough to indicate the relative importance of metabolism through nucleotides and through orthophosphate, or whether other pathways exist.

As in other organisms, part of the inorganic polyphosphate in mycobacteria is 'soluble' and part 'insoluble' in cold 5 % trichloroacetic acid depending on the degree of polymerization (Katchman & van Wazar, 1954), and the two fractions are metabolized differently. Of course, the dividing line between the two 'solubility' fractions may not correspond precisely with the metabolic dividing line, and both fractions may be metabolically inhomogeneous.

From studies on the metabolism of inorganic polyphosphate in yeast (Juni, Kamen, Spiegelman & Wiame, 1947; Juni, Kamen, Reiner & Spiegelman, 1948; Wiame, 1949; Hoffmann-Ostenhof, Klima, Kenedy & Keck, 1955), in *Corynebacterium diphtheriae* (Davis & Mudd, 1955; Sall, Mudd & Davis, 1956), in *Aerobacter aerogenes* (Duguid, Smith & Wilkinson, 1954; Smith, Wilkinson & Duguid, 1954), in Acetabularia (Stich, 1953), and in mycobacteria (Winder & Denneny, 1954, 1956) it is evident that polyphosphate (particularly 'insoluble' polyphosphate) is accumulated when the demand for high-energy phosphate drops, due to slowing of growth caused by unfavourable circumstances. When conditions again permit rapid growth polyphosphate utilization exceeds formation and its level in the cell drops. This suggests that supply and demand for phosphorus and energy control the level of inorganic polyphosphate.



Some of the work referred to above shows that this simple picture is incomplete, however, and the results in this paper raise further difficulties. Even with an unlimited supply of oxidizable substrate and phosphorus, polyphosphate accumulation does not continue indefinitely, and hence a controlling mechanism must operate when polyphosphate reaches a certain level in the cell. In *Mycobacterium smegmatis* and *M. phlei* this control is removed when substances such as THFA are added. Further, the ability of THFA to stimulate polyphosphate formation without reducing nitrogen assimilation suggests that, if polyphosphate is formed from the same pool of high energy phosphates as are used for assimilation, the compounds in this pool can be formed at a faster rate than they are required for assimilation. Why then does the start of assimilation normally (THFA absent) result in a drop in polyphosphate? This drop would appear not to be due solely to increased demand for high-energy phosphate, but to some other controlling mechanism coming into operation, and one which is also removed by THFA. This controlling factor may be the level of nucleotides available for phosphorus transfer, and THFA by reducing DNA synthesis may increase the level of nucleotides. On the other hand, THFA may possibly stimulate oxidative phosphorylation. These controls may, of course, be through cellular organization, and THFA may disturb this, which calls to mind the effect of certain organic solvents in stimulating mammalian histidine decarboxylase (Waton, 1956).

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REFERENCES

American Trudeau Society (1950). Report on the Laboratory Sub-commmittee of the Committee on Medical Research and Therapy. *Amer. Rev. Tuberc.* **61**, 274.

Cohn, W. E. & Carter, C. E. (1950). The separation of adenosine polyphosphates by ion exchange and paper chromotography. *J. Amer. chem. Soc.* **72**, 4273.

Davis, J. C. & Mudd, S. (1955). The cytology of a strain of *Corynebacterium diphtheriae*. *J. Bact.* **69**, 372.

Duguid, J. P., Smith, I. W. & Wilkinson, J. F. (1954). Volutin production in *Bacterium aerogenes* due to development of an acid reaction. *J. Path. Bact.* **67**, 289.

Hoffmann-Ostenhof, O., Kenedy, J., Keck, K., Gabriel, O. & Schönfellinger, H. W. (1954). Ein neues Phosphat-übertragendes Ferment aus Hefe. *Biochim. biophys. Acta*, **14**, 285.

Hoffmann-Ostenhof, O., Klima, A., Kenedy, J. & Keck, K. (1955). Zur Kenntnis des Phosphatstoffwechsels der Hefe. I. Quantitative Untersuchungen über das Verhalten verschiedener Phosphatfraktionen unter bestimmten Stoffwechselbedingungen. *Mh. Chem.* **86**, 604.

Juni, E., Kamen, M. D., Spiegelman, S. & Wiame, J. M. (1947). Physiological heterogeneity of metaphosphate in yeast. *Nature, Lond.* **160**, 717.

Juni, E., Kamen, M. D., Reiner, J. M. & Spiegelman, S. (1948). Turnover and distribution of phosphate compounds in yeast metabolism. *Arch. Biochem.* **18**, 387.

Katchman, B. J. & van Wazer, J. R. (1954). The 'soluble' and 'insoluble' polyphosphates of yeast. *Biochim. biophys. Acta*, **14**, 445.

Kornberg, A., Kornberg, S. R. & Simms, E. S. (1956). Metaphosphate synthesis by an enzyme from *Escherichia coli*. *Biochim. biophys. Acta*, **20**, 215.

Lieberman, I. & Eto, W. H. (1956). Inorganic triphosphate synthesis by muscle adenylate kinase. *J. biol. Chem.* **219**, 307.

Meissner, J. & Diller, W. (1953). Über die Aufnahme von Ortho- und Metaphosphate durch das *Mycobacterium tuberculosis*. *Z. Hyg. InfektKr.* **137**, 518.

Meissner, J. & Kropp, F. (1953). Über die Phosphatresorption beim *Mycobacterium tuberculosis*. *Z. Hyg. InfektKr.* **137**, 429.

Meissner, J. & Lemke J. (1955). Über die Aufnahme ^{32}P-markierter kondensierter Phosphate bei BCG- und H 37-Stämmen von *Mycobacterium tuberculosis*. *Z. Hyg. InfektKr.* **141**, 249.

Oliver, I. T. & Peel, J. L. (1956). Myokinase activity in microorganisms. *Biochim. biophys. Acta*, **20**, 390.

Ruska, H., Bringmann, G., Neckel, I. & Schuster, G. (1952). Über die Entwicklung sowie den morphologischen und zytochemischen Aufbau v. *Mycobacterium avium* (Chester). *Z. wiss. Mikr.* **60**, 425.

Sall, T., Mudd, S. & Davis, J. C. (1956). Factors conditioning the accumulation and disappearance of metaphosphate in cells of *Corynebacterium diphtheriae*. *Arch. Biochem. Biophys.* **60**, 130.

Smith, I. W., Wilkinson, J. F. & Duguid, J. P. (1954). Volutin production in *Aerobacter aerogenes* due to nutrient imbalance. *J. Bact.* **68**, 450.

Stich, H. (1953). Der Nachweis und das Verhalten von Metaphosphaten in normalen, verdunkelten und Trypaflavin-behandelten *Acetabularien*. *Z. Naturf.* **8b**, 36.

Waton, N. G. (1956). The effect of organic solvents on mammalian histidine decarboxylase. *Biochem. J.* **64**, 318.

Wiame, J. M. (1949). The occurrence and physiological behaviour of two metaphosphate fractions in yeast. *J. biol. Chem.* **178**, 919.

Winder, F. & Denneny, J. M. (1954). Metaphosphate in mycobacterial metabolism. *Nature, Lond.* **174**, 353.

WINDER, F. & DENNENY, J. M. (1955). Utilization of metaphosphate for phosphorylation by cell-free extracts of *Mycobacterium smegmatis*. *Nature, Lond.* **175**, 636.

WINDER, F. G. & DENNENY, J. M. (1956). Phosphorus metabolism of mycobacteria: determination of phosphorus compounds in some mycobacteria. *J. gen. Microbiol.* **15**, 1.

YOSHIDA, A. & YAMATAKA, A. (1953). On the metaphosphate of yeast. I. *J. Biochem. (Japan)*, **40**, 85.

(*Received* 15 *April* 1957)

Jones, A. S., Marsh, G. E. & Rizvi, S. B. H. (1957). *J. gen. Microbiol.* **17**, 586–595

The Isolation and Composition of the Nucleic Acids of *Aerobacter aerogenes*

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SUMMARY: The nucleic acids were isolated from *Aerobacter aerogenes* by extraction of mechanically disintegrated organisms with 0·14M-sodium chloride at pH 7·5. Upon subsequent isolation, about 80 % of the deoxyribonucleic acid (DNA) originally contained in the organisms was obtained, but only about 50 % of the ribonucleic acid (RNA). The loss of RNA was mainly due to enzymic degradation. By extraction of *A. aerogenes* with 1 % sodium cholate at 60°, enzymic degradation was virtually eliminated and a good yield of both DNA and RNA obtained. They were relatively undegraded and both showed evidence of the presence of hydrogen bonds. The amounts of the purine and pyrimidine bases in both types of nucleic acid were determined. The DNA and RNA from the normal streptomycin-sensitive strain of *A. aerogenes* differed significantly in base composition from the corresponding nucleic acids from a strain which had been trained to grow in 5 % (w/v) streptomycin.

For the quantitative isolation of relatively undegraded nucleic acids from living cells, two factors are of importance. First, the isolation procedure should be capable of completely extracting the nucleic acids without appreciably modifying them and secondly, enzymic degradation should be eliminated. Existing methods for the isolation of deoxyribonucleic acid (DNA) closely approach this ideal, but, since ribonucleic acid-hydrolysing enzymes are often very stable and difficult to inhibit, these conditions have not been realized in most methods for the isolation of ribonucleic acids (RNA). Thus a number of mild procedures have been developed (Grinnan & Mosher, 1951; Kay & Dounce, 1953; Kirby, 1956; Lombard & Chargaff, 1956), but in these, at some stage, usually during the initial homogenization of the tissue, there might have been enzymic degradation even at the low temperatures used by some of these workers. Other methods (Crestfield, Smith & Allen, 1955; Dorner & Knight, 1953) have employed temperatures high enough to break any hydrogen bonds which might be present in native RNA. One of the best procedures so far reported appears to be that of Hunter & Butler (1956) in which the nucleic acids were extracted from *Bacillus megaterium* with sodium dodecyl sulphate at room temperature after previous treatment of the organisms with lysozyme. In this method there was the possibility of ribonuclease action during the lysozyme treatment, although the product obtained was biologically active.

The present paper describes a method for the isolation of the nucleic acids from *Aerobacter aerogenes* under conditions in which ribonucleic acid-hydrolysing enzymes are inhibited. Both DNA and RNA have been isolated in good yield and in relatively undegraded form. The compositions of the two types of nucleic acid which have been isolated from a normal strain of *A. aerogenes* and from a strain which had been trained to grow in a high concentration of streptomycin, have been determined.

METHODS

Growth of organism

Cultures of *Aerobacter aerogenes* were grown with aeration at 37° for 24 hr. on the following medium (%, w/v): Lab Lemco, 1; Oxoid bacteriological peptone, 1; sodium chloride, 0·5; and glucose, 1. Before the addition of the glucose, the medium was adjusted to pH 9, the resulting precipitate filtered off, the filtrate adjusted to pH 7 and sterilized at 10 lb./sq.in. for 15 min. The glucose was then added aseptically.

A streptomycin-resistant strain was obtained from the normal strain by repeated subcultivation in the presence of gradually increasing concentrations of the antibiotic. The strain finally obtained grew in 50 mg. streptomycin/ml. The original strain grew at a limiting concentration of 0·001 mg. streptomycin/ml. The streptomycin-resistant strain had similar biochemical reactions to the normal strain. For the isolation of nucleic acids, both strains were grown on the same medium. Nitrogen, phosphorus, pentose and deoxypentose were estimated by methods previously used (Dutta, Jones & Stacey, 1956). Total carbohydrate was determined by the phenol-sulphuric acid method (Dubois *et al.* 1951), and protein as described by Lowry, Rosebrough, Farr & Randall (1951).

Purine and pyrimidine contents of the nucleic acids

Samples of DNA (5 mg.) were dried at 110° *in vacuo* for 2–3 hr. and then hydrolysed by heating with anhydrous trifluoroacetic acid (0·2 ml.) at 155° for 60 min. The bases were separated chromatographically on Whatman no. 1 filter paper with the solvent of Wyatt (1951), eluted and estimated spectrophotometrically.

Some difficulty was experienced in effecting the complete hydrolysis of RNA with perchloric acid (see Crosbie, Smellie & Davidson, 1953) or with trifluoroacetic acid. With the latter reagent, the presence of water (*c.* 10 %, w/v) was essential for complete hydrolysis of the pyrimidine nucleotides. However, the method of Crosbie *et al.* (1953), in which RNA is hydrolysed with 0·3 N-potassium hydroxide at 37° and the mononucleotides so produced separated by paper electrophoresis, was found to be superior. The paper electrophoresis was carried out in the apparatus of Foster (1952) as described by Foster, Jones & Rizvi (1956).

The samples of RNA prepared in this work contained considerable amounts of calcium which caused precipitation of some of the nucleotides in the presence of 0·3 N-potassium hydroxide. Until this was realized, this precipitate was centrifuged off before neutralization with perchloric acid. This gave slightly erroneous results for the proportions of the nucleotides (Cox, Jones, Marsh & Peacocke, 1956). The procedure finally adopted was to adjust the alkaline digest of the RNA to pH 1 without removing this precipitate. This gave more accurate results.

RESULTS

The nucleic acid content of Aerobacter aerogenes

Aerobacter aerogenes was grown as described above, and harvested in the Sharples supercentrifuge. The organisms were washed once with distilled water and suspended in distilled water. The suspension was divided into two equal parts, one of which was freeze-dried to give 1·892 g. dry organisms. This material was then analysed by Schmidt & Thannhauser's (1945) method (Table 1).

Table 1. *The distribution of phosphorus-containing compounds in* Aerobacter aerogenes

	Phosphorus (μmole/g. dry wt. organism)
Inorganic acid-soluble phosphorus	11·7
Organic acid-soluble phosphorus	70·2
Lipid phosphorus	73·0
Deoxyribonucleic acid phosphorus	3·3
Ribonucleic acid phosphorus	390
Phosphoprotein phosphorus	16·6
Fraction insoluble in N-sodium hydroxide	13·4

The very low value obtained for DNA was due to the difficulty experienced in centrifuging down the DNA precipitate produced by acidification of the N-sodium hydroxide digest (cf. Mitchell & Moyle, 1954). Other experiments gave a higher value for DNA, but the exact value depended upon the conditions of centrifugation. A better result was obtained by estimation of the deoxypentose by Dische's diphenylamine reaction (Dische, 1930) in a hot trichloroacetic acid extract of organisms obtained by Schneider's (1945) method. Interference with the estimation by other colour-producing substances was decreased by measuring the difference between the optical density at 600 mμ. and that at 700 mμ.

These results showed that *Aerobacter aerogenes* contained 15 % (w/w) nucleic acids (assuming a phosphorus content for the nucleic acids of 8 % (w/w)) and that 18 % of this was DNA and 82 % RNA. Recent work (Jones, Rizvi & Stacey, unpublished results) has shown that this value for RNA is too high because of the presence of other phosphorus-containing compounds in the RNA fraction. An exact determination of these was not carried out on this particular culture, but from other results it was concluded that about 25–30 % of the phosphorus in the RNA fraction was due to other phosphorus-containing substances.

Isolation of the nucleic acids from Aerobacter aerogenes

The other half of the suspension of organisms was shaken with ballotini glass balls (1·0 mm. diameter) in the presence of 0·01 M-sodium arsenate at 0° for 6–7 hr. The broken organisms were extracted at 0° for 18 hr. with 0·001 M-sodium arsenate at pH 7. The suspension was decanted from the glass balls and a 20 % (w/v) aqueous solution of cetrimide (Cetavlon, I.C.I. [Pharmaceuticals] Ltd., Manchester) added until precipitation was complete. The precipitate, which contained nucleic acids, proteins and acidic polysaccharides was centrifuged off, dissolved in M-sodium chloride and the solution diluted with three volumes of water. This procedure precipitated the nucleic acids and part of the protein but left in solution the acidic polysaccharides and the rest of the protein. Two repetitions of this precipitation procedure removed the acidic polysaccharides from the precipitate. Protein was removed from the nucleic acid-containing fraction by treatment with chloral hydrate in 2·5 M-sodium chloride as previously described (Jones & Marsh, 1954). From the equivalent of 1·892 g. freeze-dried organisms, 65 mg. of white fibrous nucleic acid (P, 8·0 %) was obtained, of which 55 % was DNA. The recovery of DNA was therefore 70 % and that of RNA only about 15–20 %.

Part of the loss of RNA was due to adsorption on the cell debris, the chloral hydrate precipitate and the chloroform-protein gels. This material was recovered by extraction with aqueous 2 % (w/v) sodium dodecyl sulphate to give 63 mg. of nucleic acid, of which 90 % was RNA. The total recovery of DNA was about 80 %, therefore, and of RNA about 50 %. The rest of the RNA had been lost because of enzymic degradation during the disintegration of the organisms. This was shown by the presence of large quantities of dialysable ultraviolet-absorbing material in the extracts of the disintegrated organisms.

The nucleic acid-hydrolysing enzymes of Aerobacter aerogenes

Organisms from a 24 hr. culture of *Aerobacter aerogenes* (2 l.) were harvested in the Sharples supercentrifuge and disintegrated by shaking a thick suspension with ballotini glass balls (1 mm. diam.) at 0° for 3 hr. the pH value being maintained at 7·5. The cell debris was removed by centrifugation at 15,000 *g* and the supernatant liquid diluted with acetate buffer to pH 6·0 and ionic strength 0·1 with respect to acetate.

A sample of this extract was diluted five times with acetate buffer (pH 6; ionic strength 0·1) and incubated at 37°. At intervals, samples (1 ml.) were removed and mixed with M-calcium chloride in ethanol (0·5 ml.). (This reagent quantitatively precipitated unhydrolysed *Aerobacter aerogenes*-RNA.) The solutions were centrifuged, the supernatant liquids diluted five times with distilled water and the optical density at 260 mμ. measured. The results (Fig. 1) indicated that there was rapid hydrolysis of the nucleic acids in the extract.

This extract was allowed to stand at 37° for 5 hr. and then dialysed. The material remaining in the cellophan bag was almost free from nucleic acid but contained an enzyme capable of hydrolysing *Aerobacter aerogenes*-RNA. The activity of this enzyme was determined against *A. aerogenes*-RNA (0·5 mg./ml.) in acetate buffer pH 6 (ionic strength 0·1) at 37°. The results (Fig. 1) showed that the enzyme was activated at 37° by 0·3 M-sodium chloride, but inhibited by 3 M-sodium chloride. It was more active at 60° than at 37°, and at 60° it was inhibited by 1 % (w/v) sodium cholate. Other results indicated that the enzyme was inhibited at 37° by 0·1 % (w/v) sodium dodecyl sulphate.

Extraction of the nucleic acids from Aerobacter aerogenes *by 1 % sodium cholate at 60°*

A suspension of *Aerobacter aerogenes* (the equivalent of 5 g. of freeze-dried organisms suspended in 150 ml. water adjusted to pH 7–7·5) was added slowly to an equal volume of 2 % (w/v) sodium cholate (pH 7–7·5) containing 0·14 M-sodium chloride, maintained at 63°. During the addition of the suspension, the temperature was not allowed to fall below 60°. The mixture was kept at this temperature for 2 hr., cooled to 0° and diluted 4–5 times by prolonged stirring at 0° with 0·14 M-sodium chloride. Cell debris was then centrifuged off at 14,000 *g* and extracted with successive portions of 0·14 M-sodium chloride (adjusted to pH 7 with sodium bicarbonate) until no more nucleic acid was extracted (10–12 washings were usually necessary). The combined extract and washings were adjusted to 0·25 M with sodium chloride, cetrimide added until precipitation was complete. The procedure was then as shown in Fig. 2.

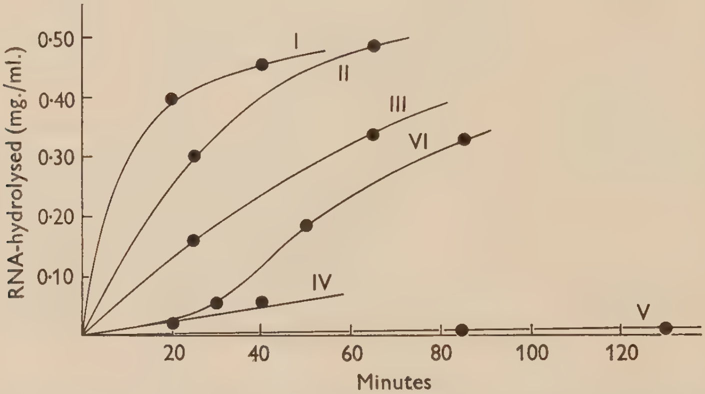
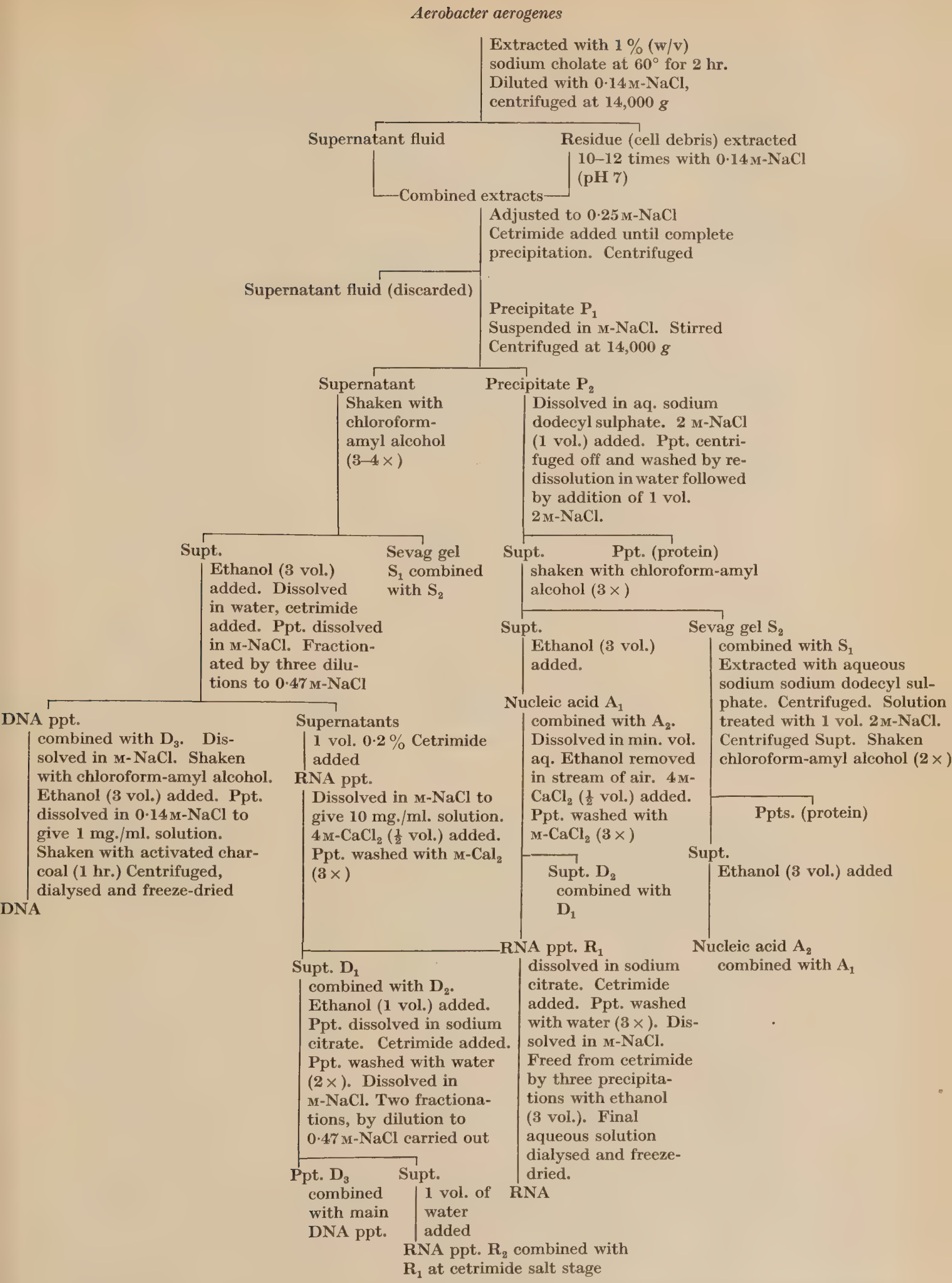


Fig. 1. Activity of the ribonucleic acid-hydrolysing enzymes of *Aerobacter aerogenes*. I–V: hydrolysis of *Aerobacter aerogenes* RNA in acetate buffer (pH 6; ionic strength 0·1); I, at 60°; II, at 37° in the presence of 0·3 M-sodium chloride; III, at 37°; IV, at 37° in the presence of 3 M-sodium chloride; V at 60° in the presence of 1 % (w/v) sodium cholate and 0·07 M-sodium chloride. VI: hydrolysis of the nucleic acids proceeding at 37° in an extract of disintegrated *Aerobacter aerogenes* in acetate buffer (pH 6; ionic strength, 0·1).

The final products had the following analysis (carried out on moist material): DNA (140 mg.); N, 11·2 %; P, 6·58 %; N/P 1·70. RNA content, 3 %; $(\epsilon)_P$, 6350. RNA (588 mg.); N, 12·3 %; P, 7·13 %; N/P, 1·72; DNA content, 1·1 % $(\epsilon)_P$, 7720.

The samples of DNA isolated by the above method had rather low phosphorus contents (8·0–8·5 % on dried material) compared with the theoretical value of 9·3 %. The RNA samples were fairly pure (theoretical phosphorus content, 8·94 %), although the presence of 2–3 % protein and the same amount of carbohydrate (other than D-ribose) was detected. The impurity in the DNA probably came from the Cetavlon, since the use of a purer product (introduced by I.C.I. Pharmaceuticals Ltd. during the progress of this work) gave nucleic acids with a phosphorus content > 9 %.



Aerobacter aerogenes

Extracted with 1 % (w/v) sodium cholate at 60° for 2 hr. Diluted with 0·14 M-NaCl, centrifuged at 14,000 *g*

Supernatant fluid

Residue (cell debris) extracted 10–12 times with 0·14 M-NaCl (pH 7)

Combined extracts

Adjusted to 0·25 M-NaCl Cetrimide added until complete precipitation. Centrifuged

Supernatant fluid (discarded)

Precipitate P_1 Suspended in M-NaCl. Stirred Centrifuged at 14,000 *g*

Supernatant Shaken with chloroform-amyl alcohol (3–4 ×)

Supt. Ethanol (3 vol.) added. Dissolved in water, cetrimide added. Ppt. dissolved in M-NaCl. Fractionated by three dilutions to 0·47 M-NaCl

DNA ppt. combined with D_3. Dissolved in M-NaCl. Shaken with chloroform-amyl alcohol. Ethanol (3 vol.) added. Ppt. dissolved in 0·14 M-NaCl to give 1 mg./ml. solution. Shaken with activated charcoal (1 hr.) Centrifuged, dialysed and freeze-dried

DNA

Supernatants 1 vol. 0·2 % Cetrimide added

RNA ppt. Dissolved in M-NaCl to give 10 mg./ml. solution. 4 M-$CaCl_2$ (½ vol.) added. Ppt. washed with M-Cal_2 (3 ×)

Sevag gel S_1 combined with S_2

Precipitate P_2 Dissolved in aq. sodium dodecyl sulphate. 2 M-NaCl (1 vol.) added. Ppt. centrifuged off and washed by redissolution in water followed by addition of 1 vol. 2 M-NaCl.

Supt. shaken with chloroform-amyl alcohol (3 ×)

Ppt. (protein)

Supt. Ethanol (3 vol.) added.

Nucleic acid A_1 combined with A_2. Dissolved in min. vol. aq. Ethanol removed in stream of air. 4 M-$CaCl_2$ (½ vol.) added. Ppt. washed with M-$CaCl_2$ (3 ×)

Supt. D_2 combined with D_1

Sevag gel S_2 combined with S_1 Extracted with aqueous sodium sodium dodecyl sulphate. Centrifuged. Solution treated with 1 vol. 2 M-NaCl. Centrifuged Supt. Shaken chloroform-amyl alcohol (2 ×)

Ppts. (protein)

Supt. Ethanol (3 vol.) added

Nucleic acid A_2 combined with A_1

RNA ppt. R_1 dissolved in sodium citrate. Cetrimide added. Ppt. washed with water (3 ×). Dissolved in M-NaCl. Freed from cetrimide by three precipitations with ethanol (3 vol.). Final aqueous solution dialysed and freeze-dried.

RNA

Supt. D_1 combined with D_2. Ethanol (1 vol.) added. Ppt. dissolved in sodium citrate. Cetrimide added. Ppt. washed with water (2 ×). Dissolved in M-NaCl. Two fractionations, by dilution to 0·47 M-NaCl carried out

Ppt. D_3 combined with main DNA ppt.

Supt. 1 vol. of water added

RNA ppt. R_2 combined with R_1 at cetrimide salt stage

Fig. 2. Procedure for the isolation of the nucleic acids from the sodium cholate extract of *Aerobacter aerogenes*.

The sugar component of the RNA was identified as D-ribose by hydrolysis with 4N-sulphuric acid followed by paper electrophoresis at pH 10 in borate buffer and paper chromatography in butanol/ethanol/water/ammonia (sp.gr. 0·880) mixture, (40:10:49:1).

The RNA was slightly fibrous although it contained only about 1 % DNA. The DNA was fibrous and upon acidification to pH 2 and readjustment to pH 7 showed a 16 % increase in optical density at 260 mμ. This increase was of a similar order to that obtained with herring roe DNA which had been isolated by a very mild procedure (Laland, Lee, Overend & Peacocke, 1954).

Composition of the nucleic acids

Samples of the DNA and RNA from a normal and a streptomycin-resistant strain of *Aerobacter aerogenes* were analysed for their purine and pyrimidine contents. Three samples were isolated from each strain and each sample was analysed in duplicate. The results are shown in Table 2. The recovery of bases was 3·9 and 3·8 mole/4 g. atom phosphorus for DNA and RNA, respectively.

Table 2. *Purine and pyrimidine contents of the nucleic acids of* Aerobacter aerogenes

	Purine and pyrimidine content (mols. per 100 nucleotides)			
	DNA		RNA	
Base	Normal strain	Streptomycin-resistant strain	Normal strain	Streptomycin-resistant strain
Guanine	28·4 ± 0·4	29·2 ± 0·4	29·6 ± 0·5	30·1 ± 0·3
Cytosine	29·2 ± 0·2	29·7 ± 0·2	24·4 ± 0·3	24·4 ± 0·3
Adenine	21·2 ± 0·2	20·8 ± 0·2	26·2 ± 0·2	25·9 ± 0·1
Thymine	21·2 ± 0·3	20·3 ± 0·3	—	—
Uracil	—	—	19·7 ± 0·6	19·5 ± 0·5

The results (Table 2) show that the composition of the DNA from the two strains differed from each other with respect to all four bases, while the RNA from the two strains differed from each other with respect to guanine, adenine and uracil. The significance of these differences was estimated by the usual methods of statistical analysis, the results of which are given in Table 3.

Table 3. *Analysis of variance for the data of Table 2*

	DNA		RNA	
Base	*F*	*P*	*F*	*P*
Guanine	12·8	< 0·01	4·45	< 0·10
Cytosine	9·94	< 0·02	—	—
Adenine	24·0	< 0·001	6·44	< 0·05
Thymine	15·8	< 0·01	—	—
Uracil	—	—	< 1	—

The differences in composition between the DNA from the normal strain and that from the streptomycin-resistant strain were highly significant. In the case of RNA the differences in the guanine and adenine contents were just significant but the difference of the uracil contents was not significant.

The statistical calculations were checked by Mr Morley-Jones, Statistician in the Agricultural Research Council Unit of Biometrical Genetics, University of Birmingham.

DISCUSSION

The isolation of the nucleic acids from *Aerobacter aerogenes* was complicated by the presence of hydrolytic enzymes which, particularly in the case of RNA, caused extensive degradation during the disintegration of the bacteria even when this was carried out at 0°. This may have been due in part to the local heating which would occur during the collisions of the glass balls. Since the hydrolytic enzymes were inhibited by 0·1 % (w/v) sodium dodecyl sulphate at 37° or by 1 % (w/v) sodium cholate at 60°, the use of these reagents for the isolation of the nucleic acids was investigated.

Aqueous 2 % sodium dodecyl sulphate readily lysed *Aerobacter aerogenes* at room temperature, and thus liberated the nucleic acids. However, since much denatured protein was also extracted and the presence of this made difficult the subsequent isolation of pure nucleic acids, this reagent was not used. Henry & Stacey (1946), however, found that sodium cholate at 60° extracted nucleic acids from Gram-positive organisms without extracting large quantities of protein. Also sodium deoxycholate has been used to extract a biologically-active DNA from pneumococci after previous heat treatment at 65° (Avery, Macleod & McCarty, 1944). Under these conditions of extraction hydrogen bonds of the type found in DNA should be left intact (Cox & Peacocke, 1956).

When applied to *Aerobacter aerogenes* this method resulted in the isolation of about 85 % of the DNA and 90 % of the RNA. The DNA was fibrous, gave viscous solutions and showed evidence of the presence of hydrogen bonds. Evidence, from titration studies, has already been presented to show that the RNA also contains hydrogen bonds (Cox *et al.* 1956).

In the DNA the amounts of guanine and adenine were equivalent to the amounts of cytosine and thymine respectively. As with most bacteria, the amount of guanine + cytosine was in excess of adenine + thymine. In the RNA, the amount of adenine + cytosine was approximately equal to that of guanine + uracil, a regularity shown by a number of ribonucleic acids (Elson, Trent & Chargaff, 1955; Elson & Chargaff, 1955).

The compositions of the DNA from the streptomycin-resistant strain showed small but significant differences from that of the DNA of the normal streptomycin-sensitive strain. Differences in the biological activity of DNA from streptomycin-resistant strains as compared with DNA from sensitive strains have been reported (e.g. Alexander & Leidy, 1953) and this demonstration of a chemical difference is of interest in this connexion. The differences in composition of the RNA from the two strains were less significant. These results may be compared with those previously obtained with *Sarcina lutea* (Dutta, *et al.* 1956) in which a difference in composition of RNA, but not of DNA, from a streptomycin-resistant strain as compared with the corresponding nucleic acids from a sensitive strain, was detected. It appears therefore that by training certain organisms to grow in the presence of streptomycin, changes in the composition of the nucleic acids can result. The commonly accepted mechanism of this change, is, in the case of streptomycin, the selection, in the presence of the drug, of a resistant mutant (Eagle & Saz, 1955).

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REFERENCES

Alexander, H. E. & Leidy, G. (1953). Induction of streptomycin resistance in sensitive *Haemophilus influenzae* by extracts containing deoxyribonucleic acid from resistant *H. influenzae*. *J. exp. Med.* **97**, 17.

Avery, O. T., MacLeod, C. M. & McCarty, M. (1944). Chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. *J. exp. Med.* **79**, 137.

Cox, R. A., Jones, A. S., Marsh, G. E. & Peacocke, A. R. (1956). On hydrogen bonding and branching in a bacterial ribonucleic acid. *Biochim. biophys. Acta*, **21**, 576.

Cox, R. A. & Peacocke, A. R. (1956). Electrometric titration of the sodium salts of deoxyribonucleic acids. Part IV. Denaturation by heat in aqueous solution. *J. chem. Soc.* p. 2646.

Crestfield, A. M., Smith, K. C. & Allen, F. W. (1955). The preparation and characterisation of ribonucleic acids from yeast. *J. biol. Chem.* **216**, 185.

Crosbie, G. W., Smellie, R. M. S. & Davidson, J. N. (1953). Phosphorus compounds in the cell. V. Composition of the cytoplasmic and nuclear ribonucleic acids of the liver cell. *Biochem. J.* **54**, 287.

Dische, Z. (1930). Über einige neue charakteristische Farbreaktionen der Thymonucleinsäure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen. *Mikrochemie*, **8**, 4.

Dorner, R. W. & Knight, C. A. (1953). The preparation and properties of some plant virus nucleic acids. *J. biol. Chem.* **205**, 959.

Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A. & Smith, F. (1951). A colorimetric method for the determination of sugars. *Nature, Lond.* **168**, 167.

Dutta, S. K., Jones, A. S. & Stacey M. (1956)., The nucleic acids of *Sarcina lutea*. *J. gen. Microbiol.* **14**, 160.

Eagle, H. & Saz, A. K. (1955). Antibiotics. *Annu. Rev. Microbiol.* **9**, 200.

Elson, D. & Chargaff, E. (1955). Evidence of common regularities in the composition of pentosenucleic acid. *Biochim. biophys. Acta*, **17**, 367.

Elson, D., Trent, L. W. & Chargaff, E. (1955). The nucleotide composition of pentosenucleic acids in different cellular fractions. *Biochim. biophys. Acta*, **17**, 362.

Foster, A. B. (1952). A simplified apparatus for filter paper ionophoresis. *Chem. & Ind.* p. 1050.

Foster, A. B., Jones, A. S. & Rizvi, S. B. H. (1956). Ionophoresis of nucleic acid components. *Chem. & Ind.* p. 914.

Grinnan, E. L. & Mosher, W. A. (1951). Highly polymerised ribonucleic acid. Preparation from liver and depolymerisation. *J. biol. Chem.* **191**, 719.

Henry, H. & Stacey, M. (1946). Histochemistry of the Gram staining reaction for micro-organisms. *Proc. Roy. Soc.* B, **133**, 391.

Hunter, G. D. & Butler, J. A. V. (1956). Stimulation by ribonucleic acid of induced β-galactosidase formation in *Bacillus megaterium*. *Biochim. biophys. Acta*, **20**, 406.

JONES, A. S. & MARSH, G. E. (1954). The deproteinisation of nucleoproteins. *Biochim. biophys. Acta*, **14**, 559.

KAY, E. R. M. & DOUNCE, A. L. (1953). The preparation of sodium ribonucleate with the use of sodium dodecyl sulphate. *J. Amer. chem. Soc.* **75**, 4041.

KIRBY, K. S. (1956). A new method for the isolation of ribonucleic acids from mammalian tissues. *Biochem. J.* **64**, 405.

LALAND, S. G., LEE, W. A., OVEREND, W. G. & PEACOCKE, A. R. (1954). The ultraviolet absorption of some degraded deoxyribonucleic acids. *Biochim. biophys. Acta*, **14**, 356.

LOMBARD, A., & CHARGAFF, E. (1956). The pentosenucleic acid of *Azotobacter vinelandii*. *Biochim. biophys. Acta*, **20**, 585.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MITCHELL, P. & MOYLE, J. (1954). The Gram reaction and cell composition: Nucleic acids and other phosphate fractions. *J. gen. Microbiol.* **10**, 533.

SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.

SCHNEIDER, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of deoxypentosenucleic acid and pentosenucleic acid. *J. biol. Chem.* **161**, 293.

WYATT, G. R. (1951). The purine and pyrimidine composition of deoxypentosenucleic acids. *Biochem. J.* **48**, 584.

(*Received* 23 *April* 1957)

Links, J., Rombouts, J. E. & Keulen, P. (1957). *J. gen. Microbiol.* 17, 596–601

The ‘Bulging Factor’, a Fungistatic Antibiotic Produced by a Streptomyces Strain, with Evidence of an Active Water-excreting Mechanism in Fungi

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SUMMARY: The ‘bulging factor’, a new antibiotic with a chemical nature related to streptothricin, is produced by a Streptomyces strain and was isolated as reineckate and picrate. A study on its mode of action resulted in the development of a plate technique for assaying bulging factor activity. Evidence is presented in support of a theory about the existence of an active water-excreting mechanism in fungi susceptible to the bulging factor.

In 1953 one of us (Rombouts, 1953) described how a rather weak antifungal antibiotic, produced by a Streptomyces strain isolated from soil in Holland, caused the hyphae of several fungi to swell in such a typical way that it can easily be distinguished by shape and size from all abnormal giant mycelial cells reported by other investigators (Wehmer, 1913; Elliot, 1917; Porter, 1924; Armitage & Verdcourt, 1947; Verdcourt, 1950, 1952; Stevenson, 1954). The product responsible for this specific deformation was named the ‘bulging factor’. The paper presented here deals with the chemical nature and mode of action of this factor.

METHODS

Cultural characteristics of the Streptomyces strain (our isolate no. S 303)

Aerial mycelium: usually abundant, white, later turning mouse grey. Sporophores produced in loose, open spirals.

Growth on defined medium agar: thin, folded, olive. Aerial mycelium scant, in fairy rings.

Growth on nutrient (glucose, Marmite, peptone) agar: thick, strongly folded. Aerial mycelium abundant. Agar turning dark brown.

Growth on starch agar: vegetative growth and aerial mycelium as on nutrient agar, but developing much slower. Hydrolysis poor. Pigment formation poor, if any.

Growth on potato glucose agar: thin, slightly folded, aerial mycelium abundant. No formation of soluble pigment.

Growth on glucose gelatine: Scant, no aerial mycelium. Liquefaction rapid around colonies. Formation of a yellow soluble pigment.

Growth on milk: dark brown ring on surface and formation of dark brown soluble pigment. Peptonization very slow.

Nitrate reduction: negative.

Temperature: optimum 26°, maximum 35°.

Production and assay of 'bulging factor'

For the production of the antibiotic Streptomyces S 303 was grown submerged in a 20 l. aerated fermentation vessel at 25° on a defined medium of the following composition: KH_2PO_4 2 g.; Na_2HPO_4 4 g.; NH_4Cl 5 g.; $MgSO_4.7H_2O$ 1 g.; $FeSO_4.7H_2O$ 20 mg.; $ZnSO_4.7H_2O$ 10 mg.; $CaCO_3$ 3·5 g.; water 1000 ml.; pH 6·8 (cf. Charney, Roberts & Fisher, 1952). The fermentation vessel was inoculated with the mycelium obtained by growing the streptomyces in shaken flasks at 25° for two days in a nutrient medium composed of: soybean meal 10 g.; liver hydrolysate 5 g.; potato starch 10 g.; KH_2PO_4 1 g.; NaCl 5 g.; $CoCl_2$ 5 mg.; water 1000 ml.; pH 6·8. The shaken flasks, in their turn, were inoculated with spores collected from agar slopes.

Bulging factor was isolated from the culture fluid when the streptomyces had grown in it for one week. The pH value was then usually 7·0. Assays of bulging factor activity were accomplished by means of the following agar plate method.

Petri dishes were provided with a 4·5 mm. thick layer of 2% (w/v) Difco agar in distilled water. After solidification the surface was evenly seeded with washed pycnospores of *Mycosphaerella pinodes* (Berk. et Blox.) Stone. These two-celled spores were produced by the Ascochyta form of the fungus growing on a nutrient agar (sucrose 50 g.; KNO_3 1 g.; KH_2PO_4 0·5 g.; $MgSO_4.7H_2O$ 0·5 g.; yeast extract 0·2 g.; agar 20 g.; water 1000 ml.; pH 6·5).

The germinating pycnospores were found to be most sensitive to bulging factor in the pink-coloured stage. They were collected from 4- or 5-day cultures and then washed twice with distilled water.

In the agar plates seeded with *Mycosphaerella pinodes* holes (0·5 cm. diam.) were made by a corkborer and filled with 2 drops of bulging factor solution and 2 drops of distilled water or solution of a substance which we wished to investigate for its influence on the bulging activity (see 'Mode of action'). In a separate hole near the periphery of the plate 2 drops of a 10^{-2}M-KCN solution were pipetted to increase the sensitiveness of the test (see 'Mode of action').

A linear relation was found between the diameter of the zone showing bulging and the logarithm of bulging factor concentration. The same type of straight-line relation has been reported for other antibiotics, as well as for growth substances (Haenel, 1954; see Cooper, 1955, for a theoretical discussion). The activity of the culture fluid was usually about 20 mm. (distance from the hole).

Extraction of bulging factor from culture filtrates

The culture fluid was adjusted to pH 1·5 with HCl. After 10 min. 60 g. charcoal (Norit P.K. 0·1–0·25 mm.) were added to 14 l. culture fluid. This suspension was stirred for 30 min. and then filtered. The pale yellow filtrate was readjusted to pH 7 with NaOH and the resulting white precipitate removed by using a filter aid (Hyflosupercel). These manipulations resulted in a partial loss of activity. The remaining active factor was adsorbed onto 150 g. Norit with continuous stirring for 30 min. The charcoal was filtered off and washed successively with: distilled water, a 1 % (w/v) solution of the disodium salt of ethylenediamine tetra-acetic acid (pH 7·5), twice with distilled water, and finally with 96 % (v/v) ethanol in water. Subsequently the bulging factor was eluted from the charcoal by stirring with 0·1 N-HCl in either methanol or 75 % (v/v) acetone in water.

An active hygroscopic precipitate was obtained by adding dry ether to the pale yellow methanolic eluate. When 75 % (v/v) acetone in water was used for the elution the eluate was neutralized with Amberlite I.R.A. 400 (OH) and then concentrated *in vacuo*. First an inactive fraction precipitated in the acetone-water solution. From the remaining concentrated solution the bulging factor was precipitated as a reineckate by adding a freshly prepared saturated solution of Reinecke salt. It was also possible to precipitate the bulging factor as an impure picrate.

RESULTS

Chemical properties

The bulging factor is very soluble in water, and not in any of the organic solvents tried. It is a basic substance. Experiments with the ion-exchanger Amberlite I.R.A. 400 (OH) indicated that acidic groups are absent. It is quite stable at pH <7 but unstable at pH >7. An aqueous solution of the factor kept nearly its full activity at pH 2 and 37° for 6 days. At pH 11, however, it soon lost activity. It is also rapidly destroyed at pH 2 at 100°. In neutral solutions at 20° with 1 % maltase or 1 % emulsin (both commercial preparations) for 16 hr. the activity remained constant. From the chemical properties we may conclude that the bulging factor has a streptothricin-like character (Peck *et al.* 1946; Bohonos *et al.* 1947; Charney *et al.* 1952; Carter, *et al.* 1954; Lindner & Wallhäuser, 1955).

Effect of the bulging factor on other micro-organisms

The bulging factor has no visible effect on the growth and multiplication of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium phlei*, *M. tuberculosis*, *Brucella abortus*, *Salmonella typhimurium*, *Haemophilus pertussis*, influenza virus, or chicken pox virus.

Mode of action

The action of certain chemicals (some of which are well known for the role they play in biochemical processes), on the width of the zone of bulging was investigated. In these experiments no KCN was added to the agar plates. Throughout these experiments a standard solution of bulging factor was used. In the holes 2 drops of this standard were mixed with 2 drops of a solution of the other chemical.

Substances with no effect on activity

No distinct effect on the diameter of the bulging zone was seen with the following substances: inorganic salts (10^{-5} to 10^{-2} M), NaCl, NaF, $NaNO_3$, NaN_3, $NaHSO_3$, $Na_2B_4O_7$, KCl, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$; vitamins (10^{-3} to 10^{-2} % w/v), thiamine, riboflavin, pyridoxine, nicotinamide, pantothenic acid; narcotics, chloroform and benzene (10^{-4} to 10^{-1} M), methanol, ethanol, and *n*-butanol (5 and 10 % w/v), urethane (10^{-4} to 10^{-1} M), dinitrophenol and malonic acid (10^{-4} to 10^{-1} M); aromatic acids, caffeic and chelidonic acid (saturated solutions), chlorogenic, protocatechuic, quinic, and shikimic acid (1 % w/v); antibiotics (0·1 % w/v), streptomycin, benzylpenicillin, terramycin (oxytetracycline); plant growth substances, indolylacetic and indolylbutyric acids (10^{-4} and 10^{-3} M) naphthylacetic acid (10^{-4} and 10^{-1} M); natural substances, glucosamine. HCl (10^{-4} to 10^{-1} M), camphor (10^{-4} to 10^{-2} M), quinine. H_2SO_4 (10^{-4} M), coumarin (10^{-4} to 10^{-2} M), adenosinetriphosphate (10^{-3} to 10^{-1} % w/v), colchicine (10^{-4} to 10^{-1} % w/v); other substances, acetone (5 and 10 % v/v), the disodium salt of ethylenediaminotetraacetic acid (10^{-3} to 1 M), helianthine (saturated solution), Reinecke salt (saturated solution), picric acid (10 % w/v), furan (10 % w/v).

Stimulation or inhibition of the activity

Potassium cyanide. Addition of KCN in concentrations between 5×10^{-5} and 5×10^{-3} M markedly increased the diameter of the bulging zone. Addition of 5×10^{-2} M-KCN caused around the hole a 10 mm.-wide zone in which neither growth of the fungus nor bulging activity was observed. Outside this toxic zone the bulging activity extended farther than in controls without KCN (e.g. to 25 and 19 mm. from the hole, respectively). KCN dosed in separate holes had the same effect; this was obviously the result of the evaporation of the formed HCN. As a result of these observations we decided to add KCN to our assay plates in order to increase the sensitivity of the tests.

Potassium tri-iodide 10^{-3} to 10^{-1} % (w/v) activated the bulging factor (e.g. control 19 mm.; with 10^{-2} % (w/v) KI_3, 23 mm.). 10 % (w/v) KI_3 caused a 15 mm. wide toxic zone with neither growth nor bulging. Beyond this zone an 8 mm. wide zone of bulging activity was observed though bulging was distinctly less pronounced than normal. Whereas low concentrations of KI_3 stimulated bulging activity and high concentrations inhibited both growth and the bulging activity, KI_3, unlike KCN, is apparently capable of exerting another special effect of unknown nature which interferes with the normal reaction of the fungus to bulging factor.

Bicarbonate ion at some concentrations suppressed bulging activity without inhibiting growth of the fungus. For instance, addition of 2 % (w/v) $NaHCO_3$ or 2 % (w/v) NH_4HCO_3 resulted in toxic zones (no growth and no bulging) of 8 mm.; outside this zone there was no bulging though growth was normal, whereas controls gave bulging zones of 19 mm.

DISCUSSION

The general picture of the abnormal development of fungal cells under the influence of the bulging factor has been described in an earlier paper by one of us (Rombouts, 1953). The typical swelling of the hypha starts always at the very tip, fully developed mycelial cells being resistant to the bulging factor. Since it is most likely that normal hyphal elongation takes place as a result of internal pressure on the young plastic cell wall at or just behind the hyphal tip, it was suggested that the bulging factor might act specifically upon the cell-wall material, or its biosynthesis, apparently by changing its directed plasticity.

However, in view of the earlier observation that in some *Fungi imperfecti* the bulging factor may exert its effect already on the germinating conidia before a proper germtube has emerged, combined with the new observations described in this paper, the following new hypothesis may be put forward. Water diffuses ('passively') into the growing young hyphae and germinating spores. The water in excess of the needs for growth is removed from the fungal cell by an energy-requiring mechanism ('actively'). Bulging factor interferes with the latter mechanism. Osmotic uptake of water is probably enhanced in primary hyphae during germination of the spores, when large molecules are broken down into smaller ones, which would increase the osmotic pressure of fungus cell-content. An exogenous source of inorganic nutrients and of a respirable carbon compound appears to be unnecessary for spore germination and the initial growth. This points to the fact that initial needs for nutrients are covered by the spore content. The KCN effect suggests that a cyanide-sensitive component of the aerobic respiration participates in the water-removing mechanism, possibly *via* a cytochrome-oxidase system. Such an energy-requiring pumping mechanism has not yet been proved or postulated to exist in fungi. Contractile vacuoles which serve the same purpose are a common feature among unicellular organisms. These have also been observed in phanerogams where they seem to play a role in sudden movements but are apparently not necessary for the maintenance of normal water balance (Weintraub, 1951; Toriyama, 1953). An active water secretion has been demonstrated in cells of several tissues of higher animals (Robinson, 1950; Wilson, 1954). These cells swell in isomotic salt solutions when they are deprived of their oxygen supply or when they are poisoned with enzyme inhibitors. The opposite, an active uptake of water, has been found in cells of certain tissues of higher plants (Reinders, 1942; Bogen, 1954).

We do not know how to explain the influence of HCO_3-ion on the bulging effect. It is unlikely that such ions are capable of a chemical decomposition of the bulging factor. The system by which the bulging factor exerts its specific action is apparently very sensitive to HCO_3-ion. It should be remembered that CO_2 is an important component in many carboxylation reactions of the living cell. In this connexion we think of the inhibitory effect of high CO_2 concentrations on the oxaloacetic acid decarboxylase in the fungus *Rhizopus nigricans* (Foster & Davis, 1949), of the possible role of CO_2 in piling up of intermediates between α-ketoglutarate and citrate (Cantino, 1951), and of the inhibition by HCO_3-ion of the respiration of excised roots (Miller & Thorne, 1956).

In the present state of knowledge it is not possible to choose between the previous and this new explanation of the bulging factor effect.

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REFERENCES

Armitage, F. D. & Verdcourt, B. (1947). The effect of sodium pentachlorphenate on the morphology of *Aspergillus niger*. *Research*, **1**, 236.

Bogen, H. J. (1954). Nichtosmotische Stoffaufnahme. *Z. Bot.* **42**, 151.

Bohonos, N., Emerson, R. L., Whiffen, A. J., Nash, M. P. & De Boer, C. (1947). A new antibiotic produced by a strain of *Streptomyces lavendulae*. *Arch. Biochem.* **15**, 215.

Cantino, E. C. (1951). Metabolism and morphogenesis in a new Blastocladiella. *Leeuwenhoek ned Tijdschr.* **17**, 325.

Carter, H. E., Clark, R. K., Kohn, P., Rothrock, J. W., Taylor, W. R., West, C. A., Whitfield, G. B. & Kackson, W. G. (1954). Streptothricin I. Preparation, properties and hydrolysis products. *J. Amer. chem. Soc.* **76**, 566.

Charney, J., Roberts, W. S. & Fisher, W. P. (1952). Pleocidin, a new antibiotic related to streptothricin. *Antibiot. & Chemother.* **2**, 307.

Cooper, K. E. (1955). Theory of antibiotic inhibition zones in agar media. *Nature, Lond.* **176**, 510.

Elliot, J. A. (1917). Taxonomic characters of the genera *Alternaria* and *Macrosporium*. *Amer. J. Bot.* **4**, 439.

Foster, J. W. & Davis, J. B. (1949). Carbon dioxide inhibition of anaerobic fumarate formation in the mold *Rhizopus nigricans*. *Arch. Biochem.* **21**, 135.

Haenel, H. (1954). Keimringbildung bei Neurospora-mutanten. *Naturwissenschaften*, **41**, 362.

Linder, F. & Wallhäuser, K. H. (1955). Die Arbeitsmethoden der Forschung zur Auffindung neuer Antibiotika. *Arch. Mikrobiol.* **22**, 219.

Miller, G. W. & Thorne, D. W. (1956). Effect of bicarbonate ion on the respiration of excised roots. *Plant Physiol.* **31**, 151.

Peck, R. L., Walti, A., Graber, R. P., Flynn, E., Hoffhine, Jr., C. E., Allfrey, V. & Folkers, K. (1946). Streptomyces antibiotics. VI. Isolation of streptothricin. *J. Amer. chem. Soc.* **68**, 772.

Porter, C. L. (1924). Concerning the characters of certain fungi as exhibited by their growth in the presence of other fungi. *Amer. J. Bot.* **11**, 168.

Reinders, D. E. (1942). Intake of water by parenchymatic tissue. *Rec. trav. bot. néerl.* **39**, 1.

Robinson, J. R. (1950). Osmoregulation in surviving slices from the kidneys of adult rats. *Proc. Roy. Soc.* B, **137**, 378.

Rombouts, J. E. (1953). Abnormal development of fungi induced by antibiotics. *Atti VI Congr. int. Microbiol.* **3**, 205.

Stevenson, I. L. (1954). Antibiotic production by Actinomycetes in soil demonstrated by morphological changes induced in *Helminthosporium sativum*. *Nature, Lond.* **174**, 598.

Toriyama, H. (1953). Observational and experimental studies of sensitive plants. I. The structure of parenchymatous cells of pulvinus. *Cytologia*, **18**, 284.

Verdcourt, B. (1950). The swelling of mould spores in the presence of hydroxy compounds. *Trans. Brit. mycol. Soc.* **33**, 323.

Verdcourt, B. (1952). The effect of certain phenolic compounds on the germination and growth of microfungi. *Mycologia*, **44**, 377.

Wehmer, C. (1913). Übergang älterer Vegetationen von *Aspergillus fumigatus* in 'Riesenzellen' unter Wirkung angehäufter Säure. *Ber. deutsch. bot. Ges.* **31**, 257.

Weintraub, M. (1951). Leaf movements in *Mimosa pudica* L. *New Phytol.* **50**, 357.

Wilson, T. H. (1954). Ionic permeability and osmotic swelling of cells. *Science*, **120**, 104.

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Decarboxylation of Neutral Amino Acids in *Proteus vulgaris*

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SUMMARY: Washed suspensions and cell-free extracts of *Proteus vulgaris* decarboxylate leucine, valine, norvaline, isoleucine, and α-amino-n-butyric acid. The system differs from most bacterial decarboxylases in being optimally active near pH 7 and in not requiring acid conditions for its formation. The system is adaptive (inducible); the presence of either leucine, valine or isoleucine will simultaneously induce decarboxylase activity against each of the five amino acids listed above. No additive effects were found when two amino acids were offered to the system simultaneously. Pyridoxal phosphate is required as coenzyme at least for valine and leucine decarboxylation; the affinity between apo- and co-enzyme is greater during decarboxylation of valine than leucine.

Gale (1940, 1946) investigated a wide variety of bacteria (over 1000 strains) for amino acid decarboxylases. While concluding that the only common amino acids attacked were tyrosine, lysine, ornithine, arginine, histidine and glutamic acid, he also cited earlier reports that amines apparently derived from certain other amino acids had been found among the products of putrefaction. The six amino acids listed above were decarboxylated by specific enzymes with certain properties in common. Their pH optima lay well to the acid side of neutrality, and maximal activity was obtained only from organisms grown in relatively acid media. Proom & Woiwod (1951) found that when *Proteus* spp. were grown on casein hydrolysate, paper chromatograms of the culture filtrates showed two new ninhydrin-positive substances. These were identified as *iso*butyl- and *iso*amylamines, arising from valine and leucine, respectively. Investigations in this laboratory (Papworth, 1951) showed that these amines were formed equally readily in media at pH 5·5, 7 or 8·5. The decarboxylase enzymes involved did not seem to fit the pattern described by Gale (1946) and were therefore investigated in more detail. While the present work was in progress, Dewey, Hoare & Work (1954) described the diaminopimelic acid decarboxylases of *Escherichia coli* and *Aerobacter aerogenes*, which also differ from Gale's enzymes in having neutral pH optima and in not requiring acid conditions for formation. The object of this study was to investigate the properties of the decarboxylase attacking valine and leucine. Preliminary reports have already appeared (King, 1953; Ekladius & King, 1956).

METHODS

Organisms. A laboratory strain of *Proteus vulgaris* (no. 10 in our list) was used (except in Table 9). No other genera were examined, as Proom & Woiwod (1951) had shown that the enzyme was confined to *Proteus* spp.

Media. Nutrient broth (Lab-Lemco, 1% (w/v); peptone, 1% (w/v); sodium chloride, 0·5% (w/v)), was obtained in the form of dried granules from Oxo Ltd., London. These were dissolved in the appropriate volume of tap-water and autoclaved for 20 min. at 10 lb./sq.in. Less active suspensions were obtained when a medium containing yeast extract and casein hydrolysate was used.

Washed suspensions. The organism was maintained on nutrient agar and subcultured every 4–6 weeks. Small bottles of nutrient broth were inoculated from a slope incubated at 37° for 6–8 hr. Roux bottles containing 150 ml. nutrient broth were then inoculated with a few drops of the young broth culture, and harvested after 15 hr. incubation at 37°. The organisms were washed twice on the centrifuge with distilled water, and then suspended in a suitable volume of buffer (0·2 M-phosphate, pH 6). The density of the suspension was determined turbidimetrically; the instrument was calibrated against suspensions whose dry weight had been determined by freeze-drying. The CO_2 output of washed suspensions in the absence of added substrate was usually negligible ($Q_{CO_2} < 1$).

Freeze-dried organisms were prepared by placing a washed suspension (suspended in water) in a small evaporating basin in a vacuum desiccator over sulphuric acid. The desiccator was evacuated until boiling followed by freezing took place, and the pressure was then reduced to 0·1 mm. Hg. These preparations remained active after several months' storage at −10°, but required addition of coenzyme for full activity (see Table 10). They provided a convenient means of storing material.

Cell-free extracts were prepared from fresh or freeze-dried organisms either by shaking with glass beads (Ballotini no. 12; King & Alexander, 1948) or by grinding with fine aluminium oxide (McIlwain, 1948). The alumina method consistently gave extracts of about the same activity as intact organisms. The ballotini extraction gave variable results since this treatment, when prolonged, tended to inactivate the enzyme. By shaking for 45 min. at 400–500 strokes/min. (2·5 in. stroke) it was usually possible to obtain extracts somewhat more active than the original organisms. Extracts prepared by either method were used the same day since appreciable inactivation took place on storage at 0° for 24 hr., although about one-fifth of the activity survived even after three weeks.

Reagents. Amino acids were from commercial sources and were examined by paper chromatography before use. Many samples of L-leucine contained valine and alanine but chromatographically pure material was obtained from Roche Products, Ltd. The L isomers were used except where otherwise indicated; DL amino acids were used where the L-compounds were not available and in some confirmatory experiments, since the synthetic racemate was less likely to be contaminated with other natural amino acids than the commercial L isomer isolated from protein hydrolysates. The pyridoxal phosphate used was a generous gift from Messrs Roche Products Ltd., through the courtesy of Dr A. L. Morrison. The solutions were stored at 0°, protected from light at all times, and discarded after 4 weeks.

Buffers used. These were: pH 4·5–5·5, potassium hydrogen phthalate + sodium hydroxide, containing 0·1 M-phthalate; pH 6–8, potassium dihydrogen phosphate + disodium hydrogen phosphate, containing 0·2 M-phosphate; pH 8·5–10, boric acid + sodium hydroxide, borate concentration 0·1 M.

Manometry. Except where otherwise stated, nitrogen-filled Warburg manometers were used at 37° and pH 6. This is not the optimum pH value for the enzyme, but was selected to permit direct observation of the course of the reaction since at this pH value CO_2 retention is small. The main compartment of the manometer flasks contained the equivalent of 15–30 mg. dry wt. organisms (or the corresponding amount of cell-free preparations), buffer, (pH 6, 0·2 M) and any other additions (e.g. coenzyme) in a total volume of 2 ml. The side-bulb contained the amino acid (final concentration 0·08 M of L isomer in most cases) dissolved in 1 ml. buffer. At pH values greater than pH 6, 0·25 ml. 2 N-HCl was placed in a second side-bulb; manometers were set up in pairs consisting of an 'experiment' flask in which the substrate was added at the beginning of the experimental period and the acid at the end, and a 'control' flask in which both acid and substrate were added at the end of the run. $Q_{CO_2}^{N_2}$ values were calculated over a period of 30 min. from addition of substrate. CO_2 output was linear except for leucine decarboxylation without added coenzyme (cf. Fig. 4, curve I): in this case Q_{CO_2} was calculated from the steady rate attained after *c.* 10 min.

Addition of coenzyme. Even in intact organisms the rate of leucine decarboxylation is limited by the amount of coenzyme available (see below). Most experiments—whether with leucine or other amino acids—were performed in the presence of sufficient coenzyme to saturate the system. In most cases pyridoxal, rather than pyridoxal phosphate, was used for reasons of economy; but the nature and amount of coenzyme added is stated in each experiment.

Analytical methods. Descending single-dimensional paper chromatography was carried out with the solvent butanol (480 ml.) + acetic acid (95 ml.) + butyl acetate (35 ml.) + water (195 ml.); amino acids and amines give blue spots on spraying with ninhydrin (0·1 %, in chloroform with 0·1 % collidine).

Ammonia was determined according to Conway (1947). When ammonia and amines were present together, the amines were determined by the colorimetric method of Ekladius & King (1957) and the ammonia estimated by subtracting this value from the total volatile base determined by Conway's (1947) method.

RESULTS

Metabolism of amino acids by Proteus vulgaris

In air, each of 18 amino acids tested was rapidly oxidized ($Q_{CO_2}^{N_2}$ values ranging from 50 to 250) by washed suspensions of *Proteus vulgaris* (strain 10). Bernheim, Bernheim & Webster (1935) and Stumpf & Green (1944) reported a very active L-amino acid oxidase in *Proteus* spp. In nitrogen in presence of 10^{-4} M pyridoxal no appreciable gas evolution was obtained with DL-alanine, glycine, L-glutamic acid, L-arginine, L-histidine, L-lysine, DL-ornithine, L-proline, L-tyrosine or L-tryptophane. L-Leucine, L-isoleucine and L-valine were decarboxylated and this is discussed in detail in this paper. A few other amino acids, viz. L-aspartic acid (cf. Meister, Sober & Tice, 1951); DL-serine (cf. Chargaff & Sprinson, 1943) and L-cystine (cf. Tarr, 1933; Kallio & Porter, 1950), yielded carbon dioxide. Ammonia was a major product in these cases; the reactions could not therefore be simple decarboxylations, and lie outside the main theme of this paper.

Most of the known bacterial decarboxylases have acid pH optima and are formed only in markedly acid media (Gale, 1940, 1946). Decarboxylases of this type, specific for glutamic acid and ornithine, have been reported in *Proteus* spp. (Gale, 1941). A search for this type of enzyme was made in organisms grown for 18 hr. at 37° in nutrient broth containing 1 % glucose but these did not yield carbon dioxide when tested at pH 4·5 with any of the 18 amino acids examined (cf. Gale, 1940). The organism does, however, possess an ornithine decarboxylase, preferentially formed in acid media and optimally active at pH 6 (Table 7, Fig. 1).

Decarboxylation of neutral amino acids

The rates of decarboxylation of various amino acids are shown in Table 1. D-Valine and D-leucine are not attacked, neither do they inhibit the decarboxylation of the L-isomers. L-Alanine and DL-α-amino-*iso*-butyric acid serve neither as substrates nor as inhibitors. When two substrates were added simultaneously no significant additive effects were observed (Table 1; see also Figs. 6*a*, 7*a*). Similar results were obtained with intact organisms. Phenylalanine was decarboxylated too slowly for additive effects to be demonstrated.

Table 1. *Decarboxylation of amino acids by cell-free extracts of* Proteus vulgaris

Substrate	$Q^{N_2}_{CO_2}$	Substrate	$Q^{N_2}_{CO_2}$
L-Valine	19	Valine + leucine	17
DL-Leucine	19	Valine + α-amino butyric acid	17
DL-Norvaline	13	Valine + isoleucine	17
DL-Isoleucine	11	Valine + norvaline	17
DL-α-amino-*n*-butyric acid	6	Leucine + norvaline	20
DL-Phenylalanine	1·5	Isoleucine + norvaline	16
Control (no substrates)	0	α-Aminobutyric acid + norvaline	8

Final concentrations of amino acids, 0·05 M: of pyridoxal phosphate, $1{\cdot}3 \times 10^{-5}$ M.

Products of decarboxylation

The amines were provisionally identified by paper chromatography (Proóm & Woiwod, 1951; Bremner & Kenten, 1951). Confirmation was obtained by isolation of the crystalline *p*-toluene sulphonamide derivatives.

iso*Butylamine from valine.* A large-scale washed-suspension experiment was carried out in two 250 ml. Erlenmeyer flasks carrying fittings for filling with nitrogen. Each flask contained 1·5 g. DL-valine and 0·9 mg. pyridoxal hydrochloride in 80 ml. of 0·2 M-phosphate buffer (pH 6). The suspension of *Proteus vulgaris* (750 mg. dry wt. in 18 ml.) was placed in the flask in a separate tube suspended by a thread. The flasks were filled with nitrogen and the suspension added to the substrate by tipping the flasks, which were then gently shaken at 37°. The reaction was stopped after 2·5 hr. by adding 2N-HCl till acid to litmus, the organisms removed by centrifugation, and the supernatant fluid made alkaline (excess NaOH) and distilled into 20 ml. 0·1 N-HCl. The distillate was made alkaline and extracted with ether (3 × 30 ml.). The ether extract was washed once with water, then acidified with a few drops of 2N-HCl and the ether distilled off. The residue was treated with 1 g. *p*-toluene sulphonyl chloride and 10% NaOH added gradually, with vigorous shaking. After addition of 20 ml. the mixture (now strongly alkaline) was gently heated on the water bath to decompose excess *p*-toluene sulphonyl chloride. On acidification to pH 3 with 2N-HCl, a bulky precipitate of N-*iso*butyltoluene-*p*-sulphonamide was formed. This was filtered off, washed with water, sucked dry, and dissolved in 1 ml. benzene on a steam bath. Light petroleum (b.p. 40–60°) was added until a slight turbidity was observed; the solution was filtered and left overnight in the refrigerator. The bulky mass of crystals which separated out was filtered off and dried *in vacuo*. Weight, 202 mg.; a yield of 220 mg. had been expected from the carbon-dioxide output, which had been measured separately in a parallel experiment in a Warburg manometer. The identity of the derivative was confirmed by determination of melting-point; by mixed melting-point with a sample prepared from authentic *iso*butylamine; and by nitrogen determination (Kjeldahl).

Table 2. *Characterization of amines as* p-*toluene sulphonamides*

Amino acid	Amine	M.p. found (°)	M.p. reference (°)	N (Kjeldahl) (%) Found	N (Kjeldahl) (%) Calc.
Norvaline	*n*-Butylamine	43	43* (1)	6·19	6·18
Valine	*iso*-Butylamine	78	78† (2)	6·16	6·18
Leucine	*iso*-Amylamine	65	65‡ (3)	5·72	5·81
Isoleucine	3-Methylbutylamine	33	—	5·76	5·81
Phenylalanine	2-Phenylethylamine	64	65–66§ (4)	5·10	5·09
α-Amino-*n*-butyric acid	*n*-Propylamine	52	52‖ (5)	6·61	6·57

* Demeny, 1931. † Wadekind, 1909. ‡ Anon, 1950.
§ Johnson & Guest, 1909. ‖ Marckwald, 1899.

The other amines were similarly identified (see Table 2). As no authentic sample of β-methylbutylamine (from *iso*leucine) was available and we could find no recorded m.p. for this compound in the literature, confirmation was obtained by elementary analysis of the *p*-toluene sulphonamide derivative (Drs Weiler and Strauss, Oxford). Found: C, 60·0%; H, 7·88%; S, 13·0%, N, 5·76%. Calculated ($C_{12}H_{18}NSO_2$), C 59·8%; H, 7·88%, S, 13·3%, N, 5·80%. An attempt was also made to characterize the amines by condensation with phenyl*iso*thiocyanate to give the corresponding alkyl-phenyl-thiourea. This was successful only in the case of *iso*amylamine (from leucine). The derivative was prepared and its identity confirmed (m.p. 102°); no depression on mixing with sample of derivative prepared from authentic *iso*amylamine; N found, 12·6%, calculated, 12·6%. Willard & Jones (1940) give the m.p. 101–102°.

Amine and carbon dioxide are produced in approximately equimolar amounts, without any appreciable production of ammonia (Table 3). Phenylethylamine production from phenylalanine was not investigated quantitatively as the amine production was too small for accurate estimation.

Table 3. *Production of amines and carbon dioxide from amino acids by* Proteus vulgaris *strain* 10

	Amine (μmole)			
	Expt.	Control	Diff.	CO_2 (μmole)
DL-α-Amino-*n*-butyric acid	34	3	31	29
L-Isoleucine	94	3	91	90
L-Leucine	60	3	57	61
L-Valine	90	3	87	88
DL-Norvaline	49	3	46	52

Manometers contained: (main flask), 32 mg. organism in 2 ml. buffer, pH 6, with 10^{-4}M-pyridoxal phosphate; (side bulb 1), 1 ml. amino acid (0·125M-leucine, isoleucine, valine; 0·25M-norvaline, aminobutyric acid); (side-bulb 2), 0·25 ml. 2N-HCl. In experimental manometers, side-bulb 1 tipped at $t=0$, side bulb 2 at $t+90$ min. In controls, both side bulbs tipped at $t=90$ min. All manometers set up in triplicate, contents pooled at end of experiment, cells removed by centrifugation, and amine determined. No significant amounts of ammonia found in any case.

Factors influencing enzyme activity

Effect of pH *value.* The pH-activity curves for the decarboxylation of valine, leucine and isoleucine are shown in Fig. 1. Excess pyridoxal (10^{-4}M) was added in each case; but experiments without added coenzyme gave similar curves. Certain precautions were necessary in determining the curve for leucine. Even in the presence of excess coenzyme, CO_2 output is sometimes more rapid in the first few minutes than later. The $Q_{CO_2}^{N_2}$ values were therefore calculated over the period 20–50 min. after addition of the leucine. At the pH values above 6, leucine was tipped in both experimental and control flasks at $t=0$, and the acid was added to stop the reaction and expel the CO_2 at $t=20$ min. in the controls and at $t=50$ min. in the experimental manometers. To ensure that output was linear over this period at the higher pH values, the course of the reaction was followed at pH 8·5 by setting up a series of manometers in which the acid was added at varying times after addition of the leucine. No excessive initial output of CO_2 was found. For comparison, the curve for the ornithine decarboxylase is included. In a number of strains of *Proteus*, Gale (1941) found this enzyme to be optimally active at pH 5·5, rather lower than our figure of 6·5.

Substrate concentration. The Michaelis constants (K_s) for the decarboxylation of leucine, norvaline, and *a*-amino-*n*-butyric acids by cell-free extracts in the presence of 2×10^{-5}M pyridoxal phosphate was 10^{-2}M in each case; for valine, a value of $2{\cdot}5\times10^{-2}$M was obtained. (Experimental data supplied by Miss B. Haughton.) For intact organisms the values were 10^{-2}M for leucine and $3{\cdot}3\times10^{-2}$M for valine (no coenzyme added).

Temperature effect. The effect of temperature is shown in Table 4. At 40° the rate of decarboxylation of both amino acids begins to decrease significantly after about 12 min. and the Q values at this temperature were calculated from the initial reaction velocity. At the lower temperatures, the reaction was linear for at least 60 min.

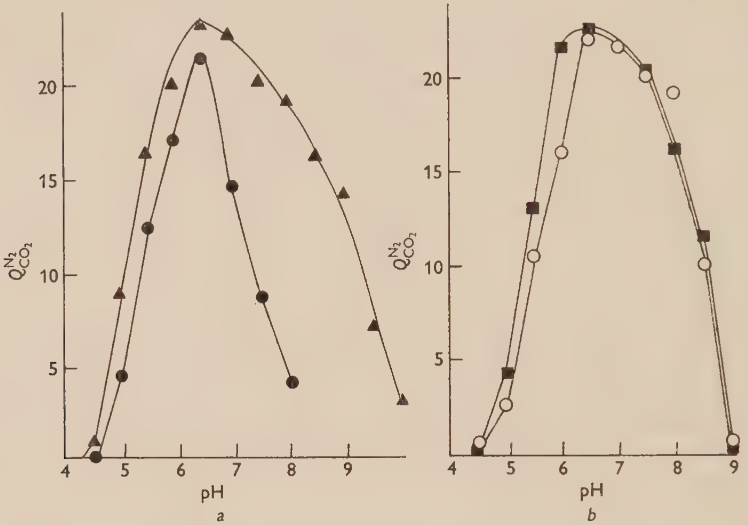

Fig. 1. pH-activity curves of decarboxylation by intact cells. *a*, ▲—▲, valine; ●—●, ornithine: *b*, ■—■, isoleucine; ○—○, leucine. All measurements (except ornithine) performed in presence of 10^{-4}M pyridoxal. Final concentration of amino acids, 0·08M. $Q_{CO_2}^{N_2}$ measured over 30 min. at 37° in nitrogen.

Table 4. *Influence of temperature on decarboxylation of valine and leucine by washed suspensions of organism*

Temp. (°)	$Q_{CO_2}^{N_2}$ [valine]	Q_{10}	$Q_{CO_2}^{N_2}$ [leucine]	Q_{10}
13	1·3	—	2·2	—
20	2·8	4·1	2·8	3·5
30	11·5	3·8	9·7	3·7
40	43·5		36·3	

All experiments carried out in presence of 10^{-4}M-pyridoxal.

Inhibitors. The action of a number of inhibitors is shown in Table 5. Two observations call for comment. (1) The decarboxylations were highly sensitive to reagents known to react with —SH groups, viz. iodoacetate, *p*-chlormercuribenzoate and mercuric chloride. (2) Decarboxylation of leucine was more sensitive than valine decarboxylation to semicarbazide and hydroxylamine, reagents known to combine with carbonyl groups. This is discussed later. Otherwise, the different decarboxylations show a fairly uniform response to any given agent.

Presence of oxygen. The effect of aeration on decarboxylation cannot be determined directly by manometery since the very rapid oxygen uptake would mask the gas output arising from decarboxylation. Amine production was

Table 5. *Sensitivity of decarboxylation to various agents*

Washed suspensions (WS) or cell-free extracts (CF) were exposed to the specific agent for 15 min. at 37° (unless otherwise indicated) and $Q_{CO_2}^{N_2}$ determined. Pyridoxal phosphate (2×10^{-5} M) added in cell-free extract experiments only.

Agent	Enzyme source	Percentage inhibition of decarboxylation				
		Leucine	Valine	Norvaline	Isoleucine	α-Amino-*n*-butyric acid
Iodoacetate (10^{-3} M)	CF	45	30	35	40	55
Mercuric chloride (10^{-4} M)	WS	100	100	—	—	—
Mercuric chloride (10^{-5} M)	WS	10	10	—	—	—
Silver nitrate (10^{-2} M)	WS	100	100	—	—	—
Silver nitrate (10^{-3} M)	WS	55	55	—	—	—
p-Chlormercuribenzoate (10^{-6} M)	CF	10	30	35	30	35
8-Hydroxyquinoline (10^{2} M)	WS	55	55	—	—	—
Potassium cyanide (10^{-3} M)	WS	80	80	—	—	—
Hydroxylamine (10^{-3} M)	WS	100	90	—	—	—
Hydroxylamine (10^{-3} M)	WS	41	0	—	—	—
Semicarbazide (10^{-3} M)	CF	90	33	84	84	90
Semicarbazide (10^{-3} M)	WS	90	55	—	—	—
Ethanol 25 % (v/v) for 5 min. (*a*)	WS	25	0	—	—	—
Ethanol 50 % (v/v) for 5 min. (*a*)	WS	100	100	—	—	—
Potassium hydrogen phthalate (0·1 M) for 10 min. (*a*)	WS	100	100	—	—	—
Na_2CO_3 (0·1 M) for 10 min. (*a*)	WS	15	33	—	—	—
Heating at 60° for 10 min.	CF	65	70	65	70	80
Storage at 0° for 3 weeks	CF	80	80	80	80	85

(*a*) Organisms centrifuged down after exposure, washed and resuspended in pH 6 buffer.

therefore used as a measure of decarboxylation. Valine and leucine were decarboxylated by washed organisms at pH 7 both in nitrogen-filled, and in air-filled manometers with KOH in the centre cup. The oxygen uptake was measured in the latter case to ensure that the substrates were not exhausted before the end of the experiment. The reaction was stopped after 60 min. by addition of acid, the organisms removed by centrifugation, and the amine in the supernatant fluids estimated. The results (Table 6) showed that decarboxylation was retarded under conditions of good aeration.

Table 6. *Effect of aeration on amine formation*

Substrate	Amine formed (μmole)	
	Aerobic	Anaerobic
Valine	16·5	28·5
Leucine	4·5	10·8
None	0·0	0·0

Washed suspensions allowed to act on leucine or valine in presence of air or of nitrogen at pH 7; acid added at end of experiment, the organisms removed and amine estimated. Duration of experiment, 60 min. Final concentrations; valine, 0·16 M, leucine, 0·08 M. No coenzyme added.

Factors affecting formation of the enzyme

Age of culture. Valine decarboxylation proceeded rapidly in young cultures but fell to a low value when active growth ceased (Fig. 2). The older cultures also showed large endogenous carbon dioxide production.

pH *value during growth.* Washed suspensions were made from organisms grown on nutrient broth buffered with phosphate at pH 5·5 (0·1 M), pH 6·5, 7·5 and 8·5 (0·05 M). These buffers kept the pH value fairly constant during growth (see Table 7). The growth rate did not vary appreciably over this

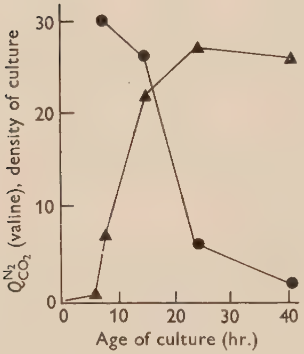

Fig. 2

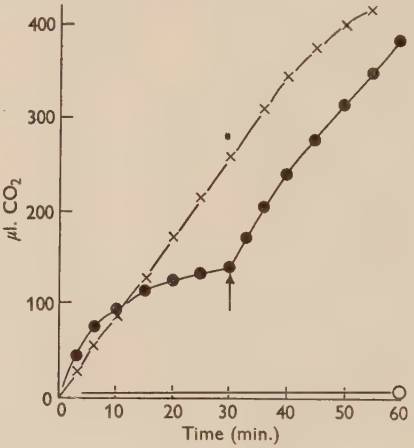

Fig. 3

Fig. 2. Effect of age of culture on valine decarboxylase activity. Organisms grown on nutrient broth at 37° for the time specified, and the growth estimated turbidimetrically washed suspensions prepared and $Q^{N_2}_{CO_2}$ determined under standard conditions with valine (final concentration 0·08 M) as substrate. ●—●, $Q^{N_2}_{CO_2}$ (valine); ▲—▲, culture density (nephelometer readings).

Fig. 3. Effect of pyridoxal phosphate on decarboxylation of leucine. Manometers contained washed suspension (35 mg. dry wt.) in main flask. Valine (0·04 M) added at $t=0$, ×—×; leucine (0·04 M) at $t=0$, pyridoxal (10^{-4} M) at $t=30$ min., ●—●; no-substrate control, ○—○.

Table 7. *Effect of* pH *value during growth on development of decarboxylase activity*

pH value of medium				
Initial	Final	$Q^{N_2}_{CO_2}$ (ornithine)	$Q^{N_2}_{CO_2}$ (leucine)	$Q^{N_2}_{CO_2}$ (valine)
(See legend)	4·5	16·2	2·1	2·1
5·5	5·8	8·5	4·7	4·1
6·5	6·8	2·4	4·8	3·4
7·5	7·6	1	5·7	5·7
8·5	8·5	1	3·8	2·5

Organisms were grown in broth buffered to the pH indicated in col. 1, and harvested after 18 hr. at 37° and washed suspensions prepared. In the first line, unbuffered broth (pH 7) containing 1 % glucose was used, the pH value falling to 4·5 during growth. The $Q^{N_2}_{CO_2}$ values were determined over a 90 min. period at pH 7; no coenzyme was added; amino acids at 0·02 M final concentration.

range; though at higher or lower pH values it declined sharply. The 'pH 4·5' results were obtained from organisms grown for 18 hr. at 37° in unbuffered nutrient broth containing 1 % glucose; the pH value fell to 4·5 during growth. It was not possible to initiate growth at this pH value. Both valine and leucine decarboxylation remained fairly constant save at the extremes of the range; ornithine decarboxylase forms a sharp contrast, being preferentially formed in acid media and thus conforming to the pattern described by Gale (1940, 1946).

Temperature. Some decarboxylases are preferentially formed at temperatures below 37° (Gale, 1940). No increase in activity was obtained however when the organisms were grown for 24 hr. at 30° instead of the usual 15 hr. at 37°.

Table 8. *Relation between decarboxylase activity and presence of amino acid in the medium during growth*

	$Q_{CO_2}^{N_2}$ when decarboxylating					
Amino acid added to medium	Leucine	Valine	Norvaline	Isoleucine	Amino-butyric acid	No substrate
None	2	2	1	0	0	0
L-Leucine	29	19	17	14	12	0
DL-Valine	13	9	8	7·5	5	0
DL-Norvaline	6	3	3	3	1·5	0
DL-Isoleucine	9	7	7	5	3·5	0
DL-Aminobutyric acid	3	2	1	2	—	0
Organisms grown on broth	37	24	23	18	13	0

Organisms were grown on defined medium with lactate as sole carbon source for 16 hr., and the amino acid indicated was added to give a concentration of 0·05 M of L-isomer. Incubation was continued for further 3·5 hr., cell-free extracts prepared and decarboxylase activity was then determined in presence of $1{\cdot}3 \times 10^{-5}$ M-pyridoxal phosphate.

Medium. Cell-free extracts prepared from organisms grown on a defined medium showed only slight activity (Table 8). The medium used contained $(NH_4)_2SO_4$, 0·8 % (w/v); NaCl, 0·5 % (w/v); KH_2PO_4, 0·2 % (w/v); Na_2HPO_4, 0·2 % (w/v); nicotinamide 1:200,000; sodium lactate, 0·8 % (w/v) in tap water. The effect of certain amino acids in stimulating enzyme formation was examined as follows. Organisms were grown for 16 hr. at 37° on the defined medium, the inoculum being prepared from a culture of *Proteus vulgaris* (strain 10) maintained on this medium. The amino acid under test was then added (final concentration 0·05 M of L-isomer). After incubation for a further period of 3·5 hr. at 37° the organisms were harvested and cell-free extracts prepared in the usual way. These were examined for decarboxylase activity with the amino acids listed in Table 8. Leucine and valine stimulated formation of decarboxylase readily; isoleucine and norvaline showed less activity. The relative rates of decarboxylation of the five amino acids investigated was independent of whether the cells were grown on broth, or on defined medium with subsequent stimulation by an 'inducer' amino acid.

Distribution of the decarboxylase. The rates of decarboxylation of valine and leucine by 15 strains of *Proteus* are given in Table 9. The following facts emerge. The decarboxylase was found in *P. vulgaris* and *P. mirabilis* but not in *P. rettgeri*; this confirms Proom & Woiwod's (1951) findings. Leucine always showed a rapid initial decarboxylation followed by a decline to a low value; valine always underwent decarboxylation at a steady rate over the 30 min. period of the experiment. In all cases the initial rate of decarboxylation was of the same order for both amino acids.

Table 9. *Decarboxylation of valine and leucine by* Proteus *strains*

	$Q_{CO_2}^{N_2}$ valine	$Q_{CO_2}^{N_2}$ leucine (initial)	$Q_{CO_2}^{N_2}$ leucine (steady)
P. vulgaris 10	14	11	2
NCTC 4175	11	16	0
NCTC 4635	5	5	0
NCTC 4636	5	3	0·5
P. vulgaris II NCTC 8311	5	5	0·5
NCTC 8313	4	6	0
P. mirabilis NCTC 60	8	12	2
NCTC 4199	7	7	2
NCTC 5887	6	8	2
P. mirabilis II NCTC 2100	16	16	4
NCTC 6790	13	10	1·5
NCTC 8309	8	7	3
P. rettgeri NCTC 5520	0	0	—
NCTC 7474	0	0	—
NCTC 7475	0	0	—

$Q_{CO_2}^{N_2}$ values determined using washed suspensions under standard conditions; valine and leucine, 0·03 M. No coenzyme added.

Coenzyme requirements. When leucine was added to a washed suspension of *Proteus*, without added coenzyme, there was a rapid initial reaction which decreased to a slower steady rate after about 10 min. Valine, norvaline, *iso*-leucine, and α-amino-*n*-butyric acid however maintained a linear CO_2 output for at least 40 min. When pyridoxal phosphate was added, leucine behaved like valine and maintained a fairly linear output (Fig. 3). The concentration of pyridoxal phosphate giving half-maximal velocity was 2×10^{-6} M (Fig. 5). Pyridoxal was equally effective; pyridoxamine required about a tenfold greater concentration. The following were inactive at 10^{-3} M; pyridoxin, adenine, adenosine-5-triphosphate, biotin, diphosphopyridine nucleotide (coenzyme I), folic acid, glutathione, calcium pantothenate, riboflavin, thiamine. Pyridoxal phosphate did not accelerate the decarboxylation (by washed suspensions) of any of the other amino acids investigated. It would seem that the washed suspensions might have contained sufficient coenzyme to saturate the apoenzyme when, say, valine was being decarboxylated, but not with leucine. This was borne out by the following observation. The decarboxylation of leucine could be stimulated to the degree attained with pyridoxal phosphate by addition to the washed suspension of boiled organisms *from the same suspension*; but to attain optimal activity, the washed organisms required

2–5 times their own weight of boiled material. (These experiments were carried out with suspensions prepared from freeze-dried organisms; otherwise, trouble arose from the large 'blank' output of gas which took place when fresh suspensions were allowed to act on boiled organisms). Organisms did not lose activity appreciably on freeze-drying, but after prolonged storage there was some loss of valine decarboxylase activity, and greater (sometimes almost complete) loss with leucine. Activity with both amino acids could be restored by adding pyridoxal phosphate, or, less effectively, by pyridoxal (Table 10).

Table 10. *Coenzyme requirements of freeze-dried organisms*

	Suspension of freeze-dried organisms		
	No addition	Addition of pyridoxal, 10^{-4}M	Addition of pyridoxal phosphate, 10^{-4}M
Expt. 1			
$Q_{CO_2}^{N_2}$ (valine)	22	26	43
$Q_{CO_2}^{N_2}$ (leucine)	4	11·5	42
Expt. 2			
$Q_{CO_2}^{N_2}$ (valine)	15	25	35
$Q_{CO_2}^{N_2}$ (leucine)	1·5	10	39

The freeze-dried material had been stored at 0° for 8 months in Expt. 1, and 2 months in Expt. 2. $Q_{CO_2}^{N_2}$ values determined under standard conditions.

When examined in the absence of added coenzyme cell-free extracts usually showed only slight activity with valine and none with leucine. Activity was restored with respect to both amino acids on adding pyridoxal phosphate. Pyridoxal, pyridoxin, and pyridoxamine, and pyridoxal + ATP were inactive. With whole organisms pyridoxal and pyridoxal phosphate were equally effective in activating leucine decarboxylation; the enzyme which phosphorylates pyridoxal is apparently lost in the preparation of the extracts. Since the decarboxylation of leucine by suspensions was initially rapid, we had to consider whether either apoenzyme or coenzyme were inactivated by the reaction with leucine. Fig. 3 shows that addition of pyridoxal restored the rapid decarboxylation of leucine, even when added after the initial reaction. The apoenzyme was, therefore, apparently fully active. This was investigated further by setting up three Thunberg tubes, each containing 5 ml. of a washed suspension of organisms (150 mg. dry wt.) in buffer (pH 6). The hollow stoppers contained, respectively, 2 ml. 0·125 M-leucine, 2 ml. 0·3 M-valine, and 2 ml. buffer. The tubes were filled with nitrogen and placed in a bath at 37°. The substrates were then added to the suspensions and the reaction allowed to proceed at 37° for 30 min. The tubes were then cooled in ice, opened, and the organisms centrifuged down in cooled tubes, the supernatant fluids being discarded. The organisms were washed once and resuspended in 5 ml. of buffer (pH 6). Each suspension was divided into two portions, one of which was examined for decarboxylase activity with both leucine and valine. No difference was found between the three suspensions. The second portion of each suspension was boiled and added to a suspension of untreated freeze-dried organisms. The decarboxylation of leucine was then examined (Fig. 4). The three boiled suspensions activated the freeze-dried suspension equally, showing that they contained similar amounts of coenzyme.

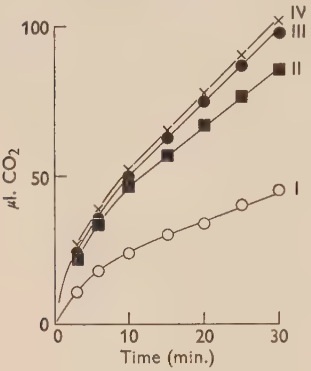

Fig. 4

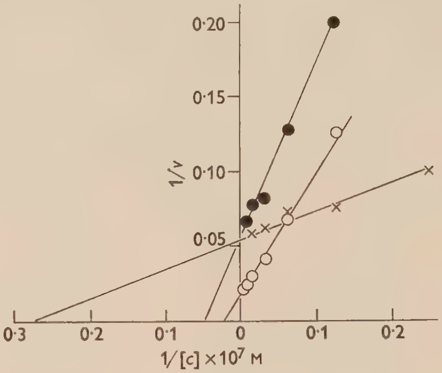

Fig. 5

Fig. 4. Effect of pretreatment of cells on coenzyme content. All manometers contained freeze-dried cells (20 mg.) in main compartment, and leucine (final concentration 0·04 M) in side-bulb, tipped at $t=0$. The main compartments contained; I, no addition; II, 30 mg. cells exposed to buffer (see text) and then boiled; III, 30 mg. cells exposed to valine prior to boiling; IV, 30 mg. cells exposed to leucine prior to boiling. I thus represents the decarboxylation of leucine without added coenzyme; II, III, and IV show the stimulation provided by the coenzyme present in the variously-treated washed cell preparations.

Fig. 5. Affinity of enzyme for coenzyme. Reciprocal plots (Lineweaver & Burk, 1934) of rate of decarboxylation (1/v) against concentration of pyridoxal phosphate (1/*C*). Leucine: (intact cells) dots, (cell-free extracts) circles. Valine (cell-free extracts). crosses. $v=Q^{N_2}_{CO_2}$, determined under standard conditions with addition of appropriate concentration of pyridoxal phosphate.

Coenzyme affinity. The results so far described suggest that combination of enzyme with coenzyme is weaker when leucine rather than valine is the substrate. This is supported by the experiments with inhibitors (Table 5); the carbonyl-fixatives, which presumably compete with the enzyme for the available coenzyme, inhibited the decarboxylation of leucine more than valine (cf. Gale, 1946). The enzyme-pyridoxal phosphate affinities were therefore determined experimentally (Fig. 5). The K_m values obtained were, for the cell-free preparations, $3{\cdot}5\times10^{-7}$ M for valine decarboxylation and 45×10^{-7} M for leucine. The affinity constant for valine decarboxylation could not be determined in suspensions of whole organisms as the enzyme was already fully saturated: but leucine decarboxylation gave a value of 20×10^{-7} M. There does not seem to be any marked tendency for the coenzyme to dissociate on preparation of the cell-free extract, as happens with some dehydrogenases (Green, 1951). The cell-free enzyme appears to bind the coenzyme about ten times more firmly when decarboxylating valine, than when leucine is the substrate. The affinity values found are of the same order, however, as those reported for

other pyridoxal phosphate systems, e.g. alanine racemase, 2×10^{-6} M (Wood & Gunsalus, 1951); tryptophanase, $2{\cdot}1 \times 10^{-6}$ M (Wood, Gunsalus & Umbreit, 1947); transaminase (mammalian muscle), 5×10^{-6} M (O'Kane & Gunsalus, 1947); tyrosine decarboxylase, 2×10^{-8} M (Umbreit & Gunsalus, 1949).

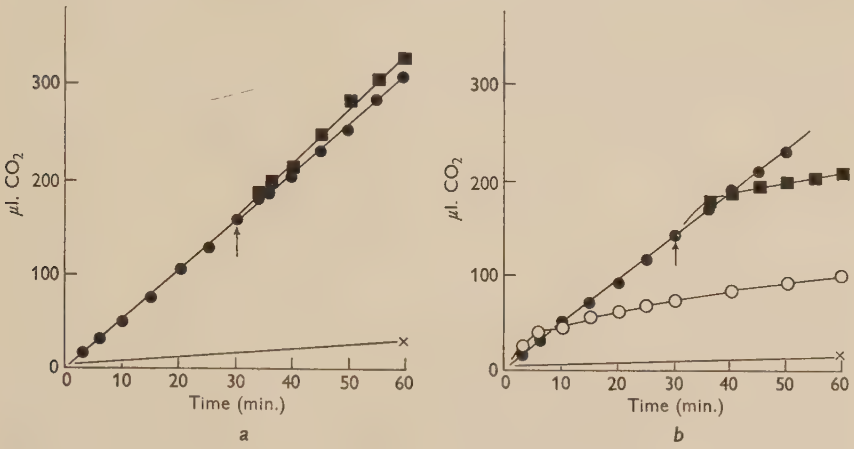

Fig. 6. Effect of leucine on valine decarboxylation by intact cells: (*a*) in presence of pyridoxal (10^{-4} M); (*b*) in absence of added coenzyme. No-substrate control, ×—×; valine (final concn., 0·04 M) added at $t=0$, ●—●; valine at $t=0$, leucine (final concn. 0·04 M) at $t=30$ min., ■—■; leucine at $t=0$, ○—○.

Competitive inhibition by coenzyme dissociation. Leucine itself has a greater affinity for the system than valine. This leads to the curious situation illustrated by Fig. 6. In Fig. 6*a*, valine was added to a suspension of organisms at $t=0$ in the presence of excess coenzyme (10^{-4} M pyridoxal) and the decarboxylation followed its usual linear course. At $t=30$ min., leucine was added from a second side-bulb (buffer added in the control) without causing any significant alteration in the rate of CO_2 output. When the experiment was repeated (Fig. 6) without addition of pyridoxal, the result was quite different. During the first 30 min. there was the usual linear decarboxylation of valine; but, on adding leucine at $t=30$ min., the rate of CO_2 evolution rapidly decreased to a slower rate more comparable with that obtained when leucine alone was being decarboxylated (cf. lower curve). The effect was more striking when a cell-free enzyme preparation was used. In the presence of 10^{-6} M-pyridoxal phosphate valine was decarboxylated rather below its optimal rate, leucine not at all. But when leucine was added to a preparation already decarboxylating valine, the reaction was completely inhibited within a few minutes (Fig. 7*b*). No such inhibition was seen when 10^{-4} M-pyridoxal phosphate was used; this concentration suffices to saturate either leucine or valine decarboxylation. In these experiments it seems that leucine, by virtue of its higher affinity, has displaced valine from the enzyme. But, when decarboxylating leucine, the enzyme appears to loosen its grip on the coenzyme. When the latter is not present in excess, the decarboxylation of *both* amino acids is partially or completely inhibited.

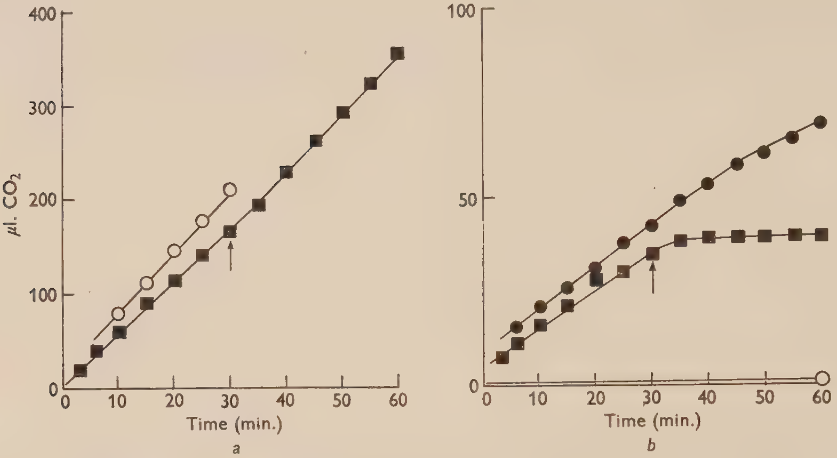

Fig. 7. (*a*) Effect of leucine on valine decarboxylation by a cell-free preparation in presence of excess (10^{-4} M) pyridoxal phosphate. ○—○, decarboxylation of leucine; ■—■, decarboxylation of valine, leucine added at $t = 30$ min. (*b*) The same experiment but with limiting concentration (10^{-6} M) of pyridoxal phosphate. ○—○, leucine decarboxylation (negligible); ●—●, decarboxylation of valine: ■—■, decarboxylation of valine inhibited by addition of leucine at $t = 30$ min.

DISCUSSION

We must first consider whether a single enzyme of broad specificity is involved, or a number of highly specific enzymes. It will be noted that valine and leucine decarboxylase activities ran approximately parallel for the series of organisms recorded in Table 9, and that both showed similar sensitivity to a variety of inhibitors and destructive agents (Table 5), except that leucine decarboxylation was more sensitive towards the carbonyl fixatives. More crucial evidence however is provided by the following observations.

(1) When the cell-free extracts were offered two amino acids simultaneously, in none of the six combinations tested was a rate of CO_2 output found greater than that which could be achieved with one substrate alone. This was true whether the substrates were added simultaneously (Table 1) or sequentially (Figs. 6, 7); whole organisms and cell-free preparations behaved alike in this respect.

(2) The decarboxylase system is adaptive (inducible) and is not formed in a simple defined medium containing no amino acids. The relative activity towards the various potential substrates does not depend upon which of them is used as inducer. Thus, the decarboxylase activity for leucine is still greater than for valine, even in extracts prepared from organisms in which the system has been induced by valine.

(3) The phenomenon of competitive inhibition by coenzyme dissociation (Figs. 6, 7). If we assume that valine and leucine decarboxylation are catalysed by separate enzymes we must interpret these results as being due to the leucine enzyme pulling the coenzyme away from the valine decarboxylase, and this is hard to reconcile with the fact that it is the *valine* system which has the

greater affinity for the coenzyme (Fig. 5). The alternative explanation, that a separate leucine enzyme acts also as a competitive inhibitor of valine decarboxylation, seems unlikely in view of the failure of leucine to inhibit valine decarboxylation when adequate coenzyme is available. These results are, however, consistent with the decarboxylation of valine and leucine being due to a single enzyme.

Although the bacterial amino acid decarboxylases described by other workers (Gale, 1940; Dewey *et al.* 1954) are highly specific enzymes, it seems difficult to maintain that we are dealing with a number of separate and specific enzymes here, in view of the failure to obtain an additive effect when more than one substrate is offered, the quantitatively non-specific action of the inducers, and the inhibition by leucine in the presence of low concentration of coenzyme. We recognize, however, that it is never possible to prove that a series of reactions is not due to separate enzymes. But we consider that the most reasonable interpretation of these results is that at least valine and leucine are decarboxylated by a common enzyme, which is possibly also responsible for the breakdown of isoleucine, norvaline, and α-amino-n-butyric acid. The existence of separate decarboxylases for these last two amino acids, which are not normally found in biological materials, would be unlikely on *a priori* grounds. The position of phenylalanine is not clear since the decarboxylation of the amino acid is too slow for convenient study.

Table 11. *Comparison of properties of certain amino and decarboxylases*

	Decarboxylase		
	Glutamic acid tyrosine, etc. (Gale, 1946)	Diamino-pimelic acid (Dewey, *et al.* 1954)	Valine, leucine, etc.
Nature of side-chain of substrate(s)	Polar	Polar	Non-polar
Optimum pH for enzyme action	Very acid	Neutral	Neutral
Optimum pH for enzyme formation	Acid	Variable	Neutral
Optimum age of culture	Mature	Variable	Young
Maximal attainable activity – $Q_{CO_2}^{N_2}$	100–1000	5–10	Up to 30
Substrate affinity approximate Ks	10^{-3} M	10^{-3} M	Valine, 0·03 M; leucine, 0·01 M

The bacterial amino acid decarboxylases hitherto described fall into two groups; one includes the six enzymes described by Gale (1946) which attack, respectively, glutamic acid, lysine, ornithine, histidine, tyrosine and arginine; the other comprises the diaminopimelic acid decarboxylases of Dewey *et al.* (1954). These enzymes are compared in Table 11 with the system now under discussion. Gale (1946), seeking a possible function for the decarboxylases, suggested that they might constitute a 'neutralization' mechanism; their formation and action at acid pH values would lead to the production of strong bases which would raise the pH to a value more suitable for growth. This suggestion is inapplicable to the system described in this paper, and we can offer no suggestion as to the part it plays in the metabolism of the cell.

The relationship of the enzyme to its coenzyme calls for some comment. The affinity of the enzyme for pyridoxal phosphate appears to depend upon the nature of the substrate undergoing decarboxylation, being greater with valine than with leucine. This is an interesting situation for which we are not aware of any precedent. It can, however, be reconciled easily with current views on enzyme action. If we assume that decarboxylation involves the formation of a complex V–E–C or L–E–C, where V and L are valine and leucine, E the enzyme and C the coenzyme, then we can see that during valine decarboxylation the dissociation of the coenzyme will be represented by the reaction $V\text{–}E\text{–}C \rightleftharpoons V\text{–}E + C$, which in the case of leucine will become $L\text{–}E\text{–}C \rightleftharpoons L\text{–}E + C$; and there is no *a priori* reason why the equilibrium constants of these two reactions should be the same.

One point remains to be explained: why should the decarboxylation of leucine start rapidly and then decline? We can only suggest that the loss of coenzyme by dissociation is a relatively slow process. On addition of leucine, several minutes elapse before the coenzyme has left the enzyme surface, and that during this period it is still able to catalyse the decarboxylation. This is consistent with the observation that in some other pyridoxal phosphate systems, the combination of coenzyme and apoenzyme proceeds quite slowly.

We are deeply grateful to Professor R. A. Morton, F.R.S., for his interest, encouragement and advice; to the Ministry of Education for a grant to one of us (C.R.S.) and to the Medical Research Council for a grant to defray expenses.

REFERENCES

Anon (1950). *Organic Reagents for Organic Analysis*, *2nd ed.*, p. 120. London: Hopkin and Williams.

Bernheim, F., Bernheim, M. L. C. & Webster, M. D. (1935). Oxidation of certain amino acids by 'resting' *Bacillus proteus*. *J. biol. Chem.* **110**, 165.

Bremner, J. M. & Kenten, R. H. (1951). Paper chromatography of amines. *Biochem. J.* **49**, 651.

Chargaff, E. & Sprinson, D. B. (1943). Studies in the mechanism of deamination of serine and threonine in biological systems. *J. biol. Chem.* **151**, 273.

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby, Lockwood and Son.

Demeny, L. (1931). Arylsulphonalkylamides. *Rec. Trav. chim. Pays-Bas*, **50**, 51.

Dewey, D. L., Hoare, D. S. & Work, E. (1954). Diaminopimelic acid decarboxylase in cells and extracts of *Escherichia coli* and *Aerobacter aerogenes*. *Biochem. J.* **58**, 523.

Ekladius, L. & King, H. K. (1956). The valine/leucine decarboxylase of *Proteus*: a study in coenzyme dissociation. *Biochem. J.* **62**, 7P.

Ekladius, L. & King, H. K. (1957). Estimation of lower aliphatic amines. *Biochem. J.* **65**, 128.

Gale, E. F. (1940). The production of amines by bacteria. I. The decarboxylation of amino acids by strains of *Bacterium coli*. *Biochem. J.* **34**, 392.

Gale, E. F. (1941). Production of amines by bacteria. 4. The decarboxylation of amino acids by organisms of the groups *Clostridium* and *Proteus*. *Biochem. J.* **35**, 66.

GALE, E. F. (1946). The bacterial amino acid decarboxylases. *Advanc. Enzymol.* **6**, 1.

GREEN, D. E. (1951). The cyclophorase system of enzymes. *Biol. Rev.* **26**, 410.

JOHNSON, T. B. & GUEST, H. W. (1909). Researches on amines: synthesis of methylphenylethylamine. *Amer. chem. J.* **42**, 340.

KALLIO, R. E. & PORTER, J. R. (1950). Metabolism of cystine and cysteine by *Proteus vulgaris* and *Proteus morganii*. *J. Bact.* **60**, 607.

KING, H. K. (1953). The decarboxylation of valine and leucine by washed suspensions of *Proteus vulgaris*. *Biochem. J.* **54**, xi.

KING, H. K. & ALEXANDER, H. (1948). The mechanical destruction of bacteria. *J. gen. Microbiol.* **2**, 315.

LINEWEAVER, H. & BURK, D. (1934). The determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658.

McILWAIN, H. (1948). Preparation of cell-free bacterial extracts with powdered alumina. *J. gen. Microbiol.* **2**, 288.

MARCKWALD, W. (1899). Die Stereochemie des Stickstoffes. *Ber. dtsch. chem. Ges.* **32**, 3508.

MEISTER, A., SOBER, H. A. & TICE, S. V. (1951). Decarboxylation of aspartic acid to α-alanine. *J. biol. Chem.* **189**, 577.

O'KANE, D. E. & GUNSALUS, I. C. (1947). Resolution and purification of glutamic-aspartic transaminase. *J. biol. Chem.* **170**, 425.

PAPWORTH, D. S. (1951). *The Nitrogen Metabolism of Bacteria.* Thesis, Liverpool.

PROOM, H. & WOIWOD, A. J. (1951). Amine production in the genus *Proteus*. *J. gen. Microbiol.* **5**, 930.

STUMPF, P. K. & GREEN, D. E. (1944). *l*-Amino acid oxidase of *Proteus vulgaris*. *J. biol. Chem.* **153**, 387.

TARR, H. L. A. (1933). The anaerobic decomposition of *l*-cystine by washed cells of *Proteus vulgaris*. *Biochem. J.* **27**, 759.

UMBREIT, W. W. & GUNSALUS, I. C. (1949). Codecarboxylase not pyridoxal-3-phosphate. *J. biol. Chem.* **179**, 279.

WADEKIND, E. (1909). Über das Verhalten ungesättigter Gruppen in quartären Ammoniumsalzen und tertiären Sulphonamiden. *Ber. dtsch. chem. Ges.* **42**, 3939.

WILLARD, W. L. & JONES, M. Z. (1940). Optical properties of phenylthiourea derivatives. *J. Amer. chem. Soc.* **62**, 2876.

WOOD, W. A. & GUNSALUS, I. C. (1951). *d*-Alanine formation: a racemase in *Streptococcus faecalis*. *J. biol. Chem.* **190**, 403.

WOOD, W. A., GUNSALUS, I. C. & UMBREIT, W. W. (1947). Function of pyridoxal phosphate. Resolution and purification of tryptophanase of *Escherichia coli*. *J. biol. Chem.* **170**, 313.

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Optical Activity of Amino Acids Formed by Reductive Amination in *Bacillus subtilis*

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SUMMARY: Suspensions of *Bacillus subtilis* form DL-alanine when incubated with pyruvate and ammonia. When leucine and valine are formed from the analogous keto acids, only the L-isomer is found. Since *B. subtilis* has a racemase active for alanine but not for valine or leucine, it is possible that the L-alanine may be the primary product and which is then racemized.

Suspensions of *Bacillus subtilis* and of some other organisms can synthesize certain amino acids from ammonia and the corresponding keto-acids (Fairhurst, King & Sewell, 1956). When alanine is formed from pyruvate by *B. subtilis*, the racemic DL-mixture is produced. The present paper gives evidence in support of this finding (mentioned by Fairhurst *et al.* 1956, but not in detail) and discusses the mechanism of the reaction.

METHODS

Details of the organism (*Bacillus subtilis*, 4) and the method of preparing washed suspensions were given by Fairhurst *et al.* (1956).

Reagents. α-Keto-*iso*-valeric acid ('ketovaline') and α-keto-*iso*-caproic acid ('ketoleucine') were prepared by condensation of the appropriate Grignard reagent (*iso*propyl- or *iso*butyl-magnesium bromide) with ethyl oxalate, and saponification of the resulting keto-acid ester (Hida, 1935; we are grateful to Dr C. Long, Aberdeen, for sending us details of this method). The keto acids were characterized by determination of bisulphite-binding capacity, and of the amount of carbon dioxide liberated on oxidation with acid potassium permanganate; by preparation of the 2:4-dinitrophenylhydrazones, and also the nitroquinoxalinol of ketovaline (Hockenhull & Floodgate, 1952). The keto acids were converted to their sodium salts for storage and use.

L-alanine was prepared by benzoylating DL-alanine (Levy & Palmer, 1942), resolving the DL-benzoylalanine by means of the strychnine salt (Pope & Gibson, 1912), and by hydrolysing the L-benzoylalanine (Pacsu & Mullen, 1940). The optical rotation agreed with published values and no oxygen uptake was observed with D-amino acid oxidase.

D-amino acid oxidase was a commercial kidney preparation (L. Light and Co., Colnbrook, Bucks). With D-alanine, D-valine and D-leucine, 0·5 mole O_2 was taken up per mole amino acid. There was no oxygen uptake with the L-isomers of these amino acids. The preparation was used for estimation of D-amino acids by the usual Warburg technique (Negelein & Bromel, 1939).

RESULTS

Biosynthesis of valine and leucine

Valine. A suspension of organisms equiv. 3·6 mg. dry wt. was obtained from 65 Roux bottles containing altogether 10 l. of nutrient broth. It was added to the following reagents at the final concentrations specified in a total volume of 400 ml.: sodium α-keto-*iso*-valerate, 0·25 M; ammonium sulphate, 0·12 M; phosphate buffer pH 7, 0·1 M. This mixture was incubated for 6 hr. at 37°. The reaction was stopped by heating for 5 min. at 100°, and the organisms removed by centrifugation. Paper chromatography showed that valine was the only amino acid present in significant amount, and controls showed that no appreciable amount of amino-N was formed when the organisms were incubated with ammonium sulphate and buffer in the absence of any keto acid. The clear supernatant, containing *c.* 300 mg. of valine (determined by the Van Slyke nitrous acid method) was now run slowly (100 ml./hr.) on to a 40 × 3·5 cm. column of Zeokarb 225 (Permutit Ltd., London) which had been ground to 60–80 mesh, treated with 2 N-HCl, and washed to remove free acid. The effluent from the column contained the free acids corresponding to the anions present, i.e. unused α-keto acid, sulphate, and phosphate. The cations (Na^+, K^+ and NH_4^+) and the valine were retained on the column which was then washed with 400 ml. distilled water and the valine selectively eluted with 0·1 N-NH_3. The effluent was collected in 10 ml. fractions and the valine located by spot-testing with ninhydrin. The first 300 ml. of effluent contained no ninhydrin-positive material; the valine was found in the following 85 ml., which was evaporated to dryness under reduced pressure to remove ammonia. The residue was taken up in 5 ml. of boiling 50 % (v/v) ethanol in water to give a solution slightly yellow in colour because of traces of material leached from the resin. Most of this coloured material was removed by repeated boiling with small amounts of charcoal. The solution was concentrated until crystallization began and on cooling valine crystallized out in the plates characteristic of this amino acid: these were recrystallized from aqueous ethanol (50 %, v/v) and dried *in vacuo*; yield 118 mg. About the same yield was obtained when 300 mg. valine was added to an artificial mixture similar in composition to the bacterial supernatant, and subsequently isolated and crystallized by the same procedure.

Leucine was obtained in exactly the same way, save that sodium a-keto-*iso*-caproate was used in place of the keto-*iso*-valerate. The supernatant fluid obtained after removal of organisms contained 350 mg. leucine; 115 mg. recrystallized material was recovered. When allowed to crystallize slowly from water, the valine and the leucine both showed the respective characteristic crystalline forms of these two amino acids; their identity and their freedom from other amino acids was confirmed by paper chromatography.

Optical activity. The valine (107 mg.) was dissolved in 2 ml. of 2 N-HCl and examined in a 2 dm. micropolarimeter tube. $\alpha_D = +2·64°$, or $[\alpha]_D + 24·7°$. Under similar conditions a sample of commercial L-valine gave $[\alpha]_D = +27·0°$; Barrow & Ferguson (1935) found +27·1° and Fischer (1906) +28·8°. The material synthesized by the organism must therefore be mainly the L-isomer but polarimetric measurements alone do not exclude the possibility that a few percent of D-valine might also be present. When 0·5 ml. of 0·05 M-solution was treated with 25 mg. of the D-amino acid oxidase at pH 8 and 37° there was no O_2 uptake. 1 % of D-isomer could have been detected.

The leucine (115 mg. in 2 ml. 2 N-HCl) gave $\alpha_D + 1{\cdot}49°$ in a 2 dm. micro-polarimeter tube, or $[\alpha]_D = +13°$. A sample of commercial L-leucine gave $[\alpha]_D + 16°$ under similar conditions; Heilbron (1934) gives $[\alpha]_D + 17{\cdot}3°$, Stoddard & Dunn (1941) $[\alpha]_D + 15{\cdot}2°$ in 6 N-HCl. (It should be noted that L-leucine is usually referred to as 'L(−)' since its rotation is negative in water, though positive in HCl.) The biological material thus gave a somewhat lower $[\alpha]_D$ than the commercial sample used; however, it is not easy to determine low rotations accurately with small amounts of material and there is also appreciable discrepancy among the published values. When examined with the D-amino acid oxidase no D-isomer was found. It is concluded that the reductive amination system produces exclusively the L-isomers of valine and leucine.

Biosynthesis of alanine

The best yield of valine and leucine from the scarce keto acid precursors was obtained with washed suspensions. For alanine it was more convenient to grow the organisms on a medium with pyruvate as main carbon source and to isolate the alanine from the culture fluid.

Ammonium pyruvate (2 %, w/v) was added to the inorganic salt medium VII of Stephenson (1939), which was adjusted to pH 7·2 and sterilized by filtration through sintered glass. This medium (250 ml. quantities) was placed in each of three flat, circular glass vessels (penicillin flasks), approx. 9 in. diameter and 4 in. deep with offset necks. Each vessel was inoculated with 12 ml. of a young broth culture of *Bacillus subtilis* 4. Maximum alanine production was reached after 3 days at 37°, when the organisms were removed by Berkfeld filtration. The filtrate (750 ml.) contained 827 mg. alanine; it was run through a cation exchange column similar to that used for the isolation of valine and the retained alanine with 0·1 N-ammonia. On evaporation of the eluate to small bulk and cooling, the alanine separated out: and after recrystallization from water, 503 mg. was obtained. This was contaminated with a small amount of yellow material from the resin which was removed by recrystallization from 80 % (v/v) ethanol. M.p. 279°; a recrystallized sample of commercial DL-alanine melted at 286°. A solution (6·5 % w/v, in 3 N-HCl) gave $[\alpha]_D < \pm 0{\cdot}2°$. In view of the possibility that racemization had taken place during the isolation procedure, a solution was prepared similar to the culture filtrate but containing authentic L(+) alanine. The alanine was recovered and isolated from this solution by the same technique, and its optical activity was retained in full; $[\alpha]_D + 14{\cdot}5°$.

Confirmation that the alanine formed by reductive amination was the racemic mixture was obtained by preparing the benzoyl derivative (Levy & Palmer, 1942) and comparing with the benzoylation products of both authentic L- and DL-alanines in respect of melting point and optical activity (Table 1).

Table 1. *Properties of benzoylalanine*

Melting points: reference values from Heilbron (1934). Polarimeter readings, $c=7{\cdot}5$ % in one equivalent of NaOH; $l=0{\cdot}5$ dm. N by Kjeldahl.

	M.p.	M.p. (reference)		% N
Authentic L-benzoylalanine	140–142°	150°	−32·5°	7·23
Authentic DL-benzoylalanine	160–161°	164–165°	±0·5°	7·33
Bacterial benzoylalanine	159–161°	—	±0·5°	7·18
Bacterial + authentic DL-benzoylalanine	160–161°	—	—	—

Alanine racemase

The formation of DL-alanine from pyruvate does not necessarily mean that this is the primary product of reductive amination. Wood & Gunsalus (1951) have reported the presence of an alanine racemase in a number of bacteria, including *Bacillus subtilis*. We therefore sought this enzyme in *B. subtilis* 4. Centrifuge tubes were set up containing, in a total volume of 2·5 ml., 0·08 M-pyrophosphate buffer, pH 8·1 (Negelein & Bromel, 1939); washed *B. subtilis* 4, 4 mg. dry wt.; pyridoxal phosphate, 0·5 μg.; L-alanine, 0·08 M. The tubes were incubated for 30 min. at 37°, then immersed in boiling water for 5 min. and the organisms removed by centrifuging. A sample of supernatant (2 ml.) was removed and placed in the main compartment of a Warburg flask with 0·5 ml. 0·2 M-pyrophosphate buffer, pH 8·1; D-amino acid oxidase (25 mg.) was placed in the side-bulb. After addition of the enzyme the oxidation was allowed to go to completion (90 min.) and an oxygen uptake of 60 μl. was recorded. This corresponds to a conversion of 3·3 μmole of L-alanine to D-alanine/mg. dry wt. organism/hr. No D-amino acid was found in a control experiment in which the L-alanine was added to the *B. subtilis* suspension at the end of the incubation period, nor in experiments in which L-valine or L-leucine were used in place of L-alanine.

DISCUSSION

It is not necessary to postulate that the enzyme synthesizing alanine from pyruvate is an exception to the rule that when a compound containing an asymmetric carbon atom is formed by an enzymic reaction, only one of the pair of enantiomorphs is produced. The organism can convert L- to D-alanine at the rate of about 3 μmole/mg./hr. The reductive amination reaction yields, under optimal conditions, 2–3 μmole alanine/mg./hr. Assuming that this is L-alanine, a rate of conversion of L- to D- of about 1–1·5 μmole/mg./hr. would be needed to yield an optically inactive end product; and this is well within the capacity of the racemase. Valine and leucine are produced exclusively as the L-isomers; the organism does not contain a racemase for these compounds. This does not necessarily prove, however, that in the case of alanine the primary product is the L-isomer, since there is so far no evidence whether alanine, valine and leucine are formed by a single enzyme or by separate enzymes.

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REFERENCES

Barrow, F. & Ferguson, G. W. (1935). The stereochemical relationships of some optically active amines and amino-acids. *J. chem. Soc.* p. 415.

Fairhurst, A. S., King, H. K. & Sewell, C. E. (1956). Studies in amino acid biogenesis; the synthesis of alanine from pyruvate and ammonia. *J. gen. Microbiol.* **15**, 106.

Fischer, E. (1906). Spaltung der α-aminoisovaleriansäure in die optisch activen Componenten. *Ber. dtsch. chem. Ges.* **39**, 2322.

Heilbron, I. M. (1934). *Dictionary of Organic Compounds.* London: Eyre and Spottiswoode.

Hida, T. (1935). Synthesis of dimethylpyruvic acid. *J. Shanghai Sci. Inst.* **1**, 201. Abstracted in *Chem. Abstr.* **30**, 5606.

Hockenhull, D. J. D. & Floodgate, G. D. (1952). *o*-Phenylenediamine and 1:2-diamino-4-nitrobenzene as reagents for α-keto acids. *Biochem. J.* **52**, 38.

Levy, M. & Palmer, A. H. (1942). The benzoylation and resolution of alanine. *J. biol. Chem.* **146**, 493.

Negelein, E. & Bromel, H. (1939). Protein der *d*-aminosäureoxidase. *Biochem. Z.* **300**, 225.

Pacsu, E. & Mullen, J. W. (1940). An improved method for the resolution of synthetic alanine. *J. biol. Chem.* **136**, 335.

Pope, W. J. & Gibson, C. S. (1912). The resolution of benzoylalanine into its optically active components. *J. chem. Soc.* **101**, 939.

Stephenson, M. (1939). *Bacterial Metabolism*, 2nd ed. p. 318. London: Longmans, Green and Co.

Stoddard, P. M. & Dunn, M. S. (1941). Quantitative investigations of amino acids and peptides. *J. biol. Chem.* **142**, 329.

Wood, W. A. & Gunsalus, I. C. (1951). D-Alanine formation; racemase in *Streptococcus faecalis*. *J. biol. Chem.* **190**, 403.

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The Effect of Spore Germination and Development on Plate Counts of Fungi in Soil

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SUMMARY: Spores of *Didymella lycopersici* and of six soil fungi were introduced separately into small quantities of soil spread in a film over a microscope slide. Direct observations were made, following which the soil from the slides was used for plate counts at stages before and after spore germination, when mycelium had developed, and after fruiting. It was found that as soon as spores germinated numbers obtained by plating decreased in the case of four of the seven fungi tested and did not increase as mycelial development progressed but reflected immediately the occurrence of sporulation.

Spores of many fungi added to sterilized soil germinate, develop a mycelium and finally form fruiting bodies. The question arises, how are these changes reflected in plate counts made at different times on inoculated soil? Although plate counts are constantly in use for the quantitative assessment of fungi in soil, no comparison of the directly observed stages of development of the fungus with the numbers of colonies obtained on plating has been made, so far as the author is aware. In connexion with an investigation of *Didymella lycopersici* a method for studying the behaviour of the fungus in soil microscopically was developed such that previously examined material could be plated. Plate counts were found to be very misleading at some stages in the ontogeny of the fungus. The study was therefore extended to six other fungi isolated from soil.

METHODS

A medium loam from one of the Cheshunt Experimental Station glasshouses was used. Tomatoes had been grown continuously on it for many years and it had been partially sterilized by steam almost annually. Spores of *Didymella lycopersici* introduced into this soil when unsteamed for a year or more slowly died out but germinated and grew when it was partially or completely sterilized. For direct observations fungi were grown in a thin layer of soil on glass slides. These slides (hereafter referred to as soil slides) were prepared by coating an area previously roughened with pumice stone with a thin layer of purified 1 % agar at 45° and immediately sprinkling it with air-dry soil from a sprinkler consisting of a boiling-tube closed by muslin. After drying, excess soil was tapped off, leaving approximately 0·1 g. distributed over an area $\frac{3}{4}$ in. × 2 in. (slides were $\frac{3}{4}$ in. × 3 in.). The thin layer of soil provided ample pore space where fungus spores and mycelium could be seen under the low or high dry objective.

The slides were placed in boiling tubes containing moist filter paper and kept in a horizontal position. They were autoclaved when desired. Spore suspension (0·1 ml.) was used to inoculate each slide; this was sufficient to moisten the soil. After inoculation the slides were incubated in a water-saturated atmosphere at room temperature. After each slide had been examined microscopically for germination and development, it was used for plate counts. It was transferred to a boiling tube containing 10 ml. water and the film of soil was rubbed off by a sterile section-lifter. The tube was then shaken, diluted appropriately and plated.

Comparisons were made between counts from soil slides and plate counts from soil in flasks (25 g. in 100 ml. flasks at 30 % moisture content calculated on an oven-dry basis). For the first two experiments Sleeth (1945) agar was employed, modified by the addition of 0·0067 % Rose Bengal and diminution of the glucose content to 0·5 % (w/v). For the third experiment Waksman's peptone glucose agar for fungi (1928) was used. Counts of *Didymella lycopersici* did not differ on the two media.

Cultures of the fungi for the third experiment had been isolated from the soil used and maintained for about a year on Czapek-Dox agar. The strain of *Didymella lycopersici* came from diseased tomato stems, having been isolated five years previously and since then grown on Czapek-Dox agar.

RESULTS

Comparisons in unsteamed and sterilized soil. Nine flasks of soil and 9 soil slides were autoclaved for 2 hr. at 126°; an equal number of slides was not sterilized. Slides were inoculated with 50,000 spores of *Didymella lycopersici* in 0·1 ml. of water, and flasks with $12{\cdot}5 \times 10^6$ spores in 1 ml. of water. Three slides and three flasks of each series were plated immediately, three slides and flasks of each 24 hr. later, and the remainder after 4 days of incubation. Before plating, all except the initial slides were examined under the microscope. The results are shown in Table 1.

Table 1. *Numbers of colonies from plate counts and appearance of* Didymella lycopersici *in sterilized and unsterilized soil*

Duration of incubation (days)	Sterilized soil			Unsterilized soil		
	Percentage original number (av. 3 replicates)			Percentage original number (av. 3 replicates)		
	Flasks	Slides	Appearance of fungus (slides)	Flasks	Slides	Appearance of fungus (slides)
1	**1·7**	**5·5**	93 % germination. Germ tubes still short	105	97	No spores germinated
4	8·3	14·0	Mycelium extensively developed over entire soil area	109	84	No spores germinated

Figures in black are significantly different from previous day (*T* tests).

Plate counts from sterilized soil made one day after inoculation, whether of flasks or slides, showed a large decrease in the number of *Didymella lycopersici*, but direct observation showed that most of the spores had germinated and begun vigorous development. On the other hand, where spores did not germinate, in unsterilized soil, there was no significant change in the quantitive estimate of *D. lycopersici* present.

Comparisons in sterilized and partially sterilized soil. Eighteen flasks of soil and 18 soil slides were autoclaved for 2 hr. at 126°; 18 flasks and 18 slides were autoclaved for 5 min. at 115°. The flasks were inoculated as for the first experiment, and three slides and three flasks of each treatment were plated immediately and then after 1, 2, 4, 15 and 29 days of incubation. Before plating all except the initial slides were examined under the microscope (Table 2).

Table 2. *Numbers of colonies and appearance of* Didymella lycopersici *in sterilized and partially sterilized soil*

	Sterilized soil			Partially sterilized soil		
Duration of incubation (days)	Percentage original number (av. 3 replicates)		Appearance of fungus (slides)	Percentage original number (av. 3 replicates)		Appearance of fungus (slides)
	Flasks	Slides		Flasks	Slides	
1	**15·6**	**47·2**	95 % germination. Germ tubes still short	**32·9**	**43·8**	92 % germination. Germ tubes still short
2	11·0	55·1	Mycelium developing vigorously	**64·4**	**29·2**	Mycelium developing vigorously. A few bacteria present
4	13·7	83·1	Mycelium covering slide	35·6	28·1	Mycelium covering slide
15	**2051**	**11,800**	Mycelium as at 4 days. A total of 37 pycnidia with spores seen	24·8	34·8 **1292***	Mycelium as at 4 days. Bacteria profuse. No pycnidia seen
29	**6395**	13,400	As at 15 days. 27 pycnidia seen	**52·0**	24·7 **329†**	As at 15 days

Figures in black are significantly different from the previous day (*T* test).
* Two slides had low numbers on plating, one gave 1292 %.
† One slide gave 24·7 % on plating, the mean of the others was 329 %.

As in the first experiment germination and growth of the spores caused a decrease in numbers, and despite profuse growth the original value for the population was not regained until sporing took place. Development on slides was more rapid than in flasks, and on the partially sterilized slides some sporing appeared to have occurred, although no pycnidia were observed. The examination for pycnidia consisted in following the high-power field across each slide five times at intervals covering the slide length. Such an examination might easily miss the presumably few (by comparison with the sterilized slides) pycnidia present. The slight sporing on the partially sterilized slides as opposed to flasks might be due to the heat treatment having a more drastic effect on 0·1 g. soil than on 25 g. bulk soil.

Effect of germination and development of other fungi on plate counts. Soil slides were prepared and autoclaved for 2 hr. at 126°. Spores of various fungi were added at the rate of approximately 50,000/slide. Three of each set

Table 3. *Numbers of colonies and appearance of soil fungi in sterilized soil on slides*

Fungus	1 day				4 days		
	No. of replicates	Percentage original number	Significance*	Appearance of fungus	Percentage original number	Significance*	Appearance of fungus
Didymella lycopersici	3	**52·2**	—	99·1 % germination. Growth well under way	**9·7**	—	Mycelium extensively developed. No spores
Penicillium sp.	3	127·3	—	80 % germination. Germ tubes still short	135·3 **477**	—	Mycelium extensively developed. Sporing seen on slide giving 477 plate count
Cephalosporium sp.	3	98·9	—	79·5 % germination. Growth well under way	**643**	—	Mycelium extensively developed. Sporing seen on all slides
Cladosporium herbarum	3	87·5	—	5·2 % germination. Germ tubes short	**10,790**† **3,784**	—	Mycelium extensively developed. Profuse sporing
Mucor mucedo	3	50·0	0·1 %	98 % germination. Much growth	278	0·1 %	Profuse growth; sporangia present
	5	55·9		97 % germination. Considerable growth	336		Profuse growth; sporangia present
Peziza sp.	3	45·6	0·1 %	100 % germination. Slide covered with growth	111	Not significant	Extensive growth with a few spores
	5	9·4		100 % germination. Much growth of mycelium	29·0		Profuse growth
Trichoderma viride	3	82·8	5 %	31 % germination. Tubes short	408	0·1 %	Mycelium well developed. Sporing occurring
	5	77·9		3·8 % germination. Germ tubes very short	2075		Much growth. Sporing

Figures in black are significantly different from previous plating.

* Level of significance for difference from previous plating as estimated by an analysis of variance for the two trials.

† 10790 % = 1 slide, 3784 % = av. of other two which were close together.

of slides were plated immediately and three of each set of slides after 1 and 4 days of incubation. Each slide was examined before plating, as before. The experiment was repeated with five replicates in the case of three of the fungi. The results are shown in Table 3. A further test was carried out in flasks of sterilized soil to see whether in bulk soil there was also an apparent decrease in the quantity of *Mucor mucedo*, *Peziza* sp. and *Trichoderma viride* after short incubation. There were five replicate flasks of each fungus for each plating, and the flasks were plated immediately after inoculation and after 1 and 4 days of incubation; the results are shown in Table 4.

Table 4. *Numbers of colonies of three fungi in sterilized soil in flasks*

Fungus	1 day (% original number)	4 days (% original number)
Mucor mucedo	**6·8**	**2545**
Peziza sp.	**7·7**	**285**
Trichoderma viride	**69·4**	**3540**

Figures in black are significantly different from previous plating.

The plate counts of flasks 1 and 4 days after inoculation give results very similar to the plate counts of slides. It appears probable that the decrease in numbers of the three fungi at 1 day and the great increase at 4 days are related as in soil slides to the germination of the spores inoculated and to the production of new spores.

It would be of interest to extend this type of study to a much larger number of soil fungi. Apart from *Didymella lycopersici* two of the six fungi investigated (*Penicillium* sp. and *Cephalosporium* sp.) appeared to possess mycelial properties which rendered them resistant to the plating process so that there was no decrease in the plate count when a high proportion of the spores had germinated. Two other organisms, *Mucor mucedo* and the *Peziza* sp., behaved similarly to *Didymella lycopersici* in that germinated spores and mycelium appeared to be relatively much more susceptible to the plating process than ungerminated spores. The behaviour of *Cladosporium herbarum* requires more work for its understanding. The decrease in the number of *Trichoderma viride* colonies after 1 day of incubation is less than that found for the other fungi and is not clearly related to spore germination. In the trial with five replicates (Table 3) there was a decrease of 22 % at 1 day compared with the original number and only 3·8 % spore germination. After 2 days, the numbers obtained on plating had not changed but germination was 70·4 %. It is possible that in the case of *Trichoderma viride* spores about to germinate are more susceptible to damage and elimination in the plating process than dormant spores.

DISCUSSION

The work reported here shows that colony counts of *Didymella lycopersici* are not a suitable measure of the development of the mycelium of this fungus in sterilized soil. With some fungi a given number of spores produce a far higher colony count on plates than the extensive mycelium which results from their germination and development. This may partly be due to failure to break up the mycelium during shaking, but certainly is partly the result of destruction of material in plating, for germinated spores not yet joined in a mycelial weft show this diminution.

Plate counts have long been held to underestimate the amount of mycelium in unsterilized soil. This supposition is supported by Warcup's direct examination of colonies in plates for their origin (1955). He did not find any which derived from hyphae, although in direct microscopic methods for estimating the quantity of fungi present in the soil the most common units are hyphal fragments (e.g. Skinner, Jones & Mollison, 1952; McLennan & Ducker, 1954). Presumably the count of germinated spores and mycelium would be decreased by plating in unsterilized soil, as in sterilized soil. Thus, if plate counts are mainly of spores and direct counts are mainly of hyphae, it is not surprising the two are not correlated when measured in connexion with changes in temperature, season, etc., for mycelium and spores would not be expected to vary together in nature.

In the early work of McLennan (1928) it appeared that the mycelial development of fungi in sterilized soil was not accompanied by great changes in plate counts but that sudden increases took place when sporing began. This has been confirmed by the work reported here, for in every case in which there were substantial increases in plate counts sporing was observed (with the exception of three slides of partially sterilized soil + *Didymella lycopersici*, discussed in connexion with the second experiment). The plate count may be regarded as a valuable method where interest is primarily in spores. For example, there are many papers reporting on changes in number of fungi introduced as spore suspensions into non-sterile soils (e.g. Katznelson, 1940; Rombouts, 1953; Park, 1955). It must be noted, however, that decreases such as are almost always recorded in these papers can only certainly be ascribed to death of the spores when it has been ascertained by some direct means that the fungus has not germinated.

REFERENCES

Fred, E. B. & Waksman, S. A. (1928). *Laboratory Manual of General Microbiology.* New York: McGraw Hill.

Katznelson, H. (1940). Survival of micro-organisms introduced into soil. *Soil Sci.* **49**, 283.

McLennan, E. (1928). The growth of fungi in soil. *Ann. appl. Biol.* **15**, 95.

McLennan, E. I. & Ducker, S. C. (1954). The ecology of the soil fungi of an Australian heathland. *Austr. J. Bot.* **2**, 220.

Park, D. (1955). Experimental studies on the ecology of fungi in soil. *Trans. Brit. Mycol. Soc.* **38**, 130.

Rombouts, J. E. (1953). The micro-organisms in the rhizosphere of banana plants in relation to susceptibility or resistance to Panama disease. *Plant & Soil*, **4**, 276.

Skinner, F. A., Jones, P. C. T. & Mollison, J. E. (1952). A comparison of a direct and plate counting technique for the quantitative estimation of soil micro-organisms. *J. gen. Microbiol.* **6**, 261.

Sleeth, B. (1945). Agar medium and technique for isolating *Pythium* free of bacteria. *Phytopathology*, **35**, 1030.

Warcup, J. H. (1955). On the origin of colonies of fungi developing on soil dilution plates. *Trans. Brit. mycol. Soc.* **38**, 298.

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The Mineral Nutrition of *Rhizopus stolonifer*

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SUMMARY: A series of experiments with culture media was carried out dealing with: (1) the action of different salts and their interaction in the medium; (2) the relation of the nitrogen source to the action of the salts; (3) the effect of the carbon source; (4) the proportionate influence of the salts in the medium; (5) the rate of growth. The results showed that this fungus grew well at 26° with an initial pH value of 7·0–8·0 and a growth period of 6–7 days. Statistical analysis of the results showed: (*a*) that under the given conditions an aqueous medium containing: 4% (w/v) glucose; 0·048 M-K_2HPO_4; 0·012 M-$MgSO_4.7H_2O$; 0·025 M-$(NH_4)_2SO_4$; 2 p.p.m. Zn, Fe and Mn, produced optimal growth measured as mg. dry wt.; (*b*) a balance did not exist between KCl, K_2HPO_4 and $MgSO_4$; (*c*) a balance did not exist between the concentration of glucose and $(NH_4)_2SO_4$; (*d*) a balance existed between K_2HPO_4 and $(NH_4)_2SO_4$; (*e*) this fungus could only utilize KNO_3 or $Ca(NO_3)_2$ to a negligible extent, while the best source found for nitrogen was $(NH_4)_2SO_4$; the most available phosphorus source was K_2HPO_4; (*f*) the rate of growth followed a sigmoid curve in a nutritionally optimal solution but in the minimal solution used the onset of decline in dry weight was delayed; (*g*) the importance of a balanced solution is that in suitable media the buffering action of the phosphate controls the onset of acidity inimical to growth.

The object of these experiments was to study the effect of the mineral constituents of the medium in which *Rhizopus stolonifer* Ehren. was grown. They were designed to show statistically whether or not a balance between the salts was necessary in order to produce a maximum yield of dry wt. mycelium. Such experiments were first carried out in this form by Talley & Blank (1941) working with the parasitic fungus *Phymatotrichum omnivorum* and they reported that a balance between the salts was necessary for high mycelial yield. Fothergill & Raine (1954), using complementary strains of *Mucor heimalis*, reported that balance alone was not of overriding importance in the growth of the fungus. Fothergill & Ashcroft (1955) with *Venturia inaequalis*, a parasite, found that a proper balance between the major mineral constituents of the medium was necessary.

METHODS

The strain of *Rhizopus stolonifer* Ehren. used in the experiments described below was isolated from the atmosphere of Newcastle upon Tyne. Single spore cultures were kept as stocks in 6 in. × $\frac{3}{4}$ in. test tubes on potato glucose agar, usually in a refrigerator at 2°. The morphological and cultural characters of the fungus were not affected by subcultivation during the experimental period. In the experiments on nutrition inoculations were made into a final volume of 30 ml. sterile culture fluid in Hysil Erlenmeyer flasks (150 ml.) plugged with cotton wool and incubated at 26°. Sterile phosphate solution was added aseptically from a glass syringe after autoclaving in order to avoid any possible precipitation. The inoculum was obtained as a suspension of spores and mycelial fragments by adding 10 ml. sterile distilled water to a suitable agar slope, rotating and decanting off the liquid; 1 ml. was then added to each culture flask of liquid medium. It was found by experience that the exact amount of the inoculum did not appreciably affect growth within limits.

Each experiment was carried out in five replicates and pH measurements were taken before and after the experiments using a Cambridge pH meter. After the growing period the mycelia were filtered on to tared Whatman No. 5 filter papers, dried at 85° for 24 hr., cooled in a desiccator and weighed; results were thus expressed as mg. dry weight. For preliminary experiments the following basal defined liquid medium *A* was used: 4 % (w/v) glucose; 0·008 M-K_2HPO_4; 0·003 M-$MgSO_4.7H_2O$; 0·0125 M-$(NH_4)_2SO_4$; Zn, Mn and Fe at 2 p.p.m. Preliminary experiments with this medium indicated that the optimum temperature for maximum mycelial growth was 26°, although the fungus grows well from 16° up to this temperature; an onset of decline in dry weight begins on the 6th to 7th day. Robbins (1924) stated that the optimum pH value for this fungus in liquid was 5·5, but experiments with our strain showed that any pH value between 7 and 8 was optimal; a real decrease in mycelial dry weight occurred at pH 6 or less. Experiments also showed that *Rhizopus stolonifer* grew negligibly (7–9 mg. dry wt./mycelium) when KNO_3 or $Ca(NO_3)_2$ were used, thus confirming Robbins's (1937) statements. Asparagine and $(NH_4)_2SO_4$ are good sources of nitrogen. With the 7-day growth period used in these experiments phosphate had to be supplied as orthophosphate and not as pyro- or meta-phosphate because these two latter forms gave a distinct lag in growth. Experiments were carried out to determine the concentration of each salt in the medium which produced a high dry weight of mycelium.

RESULTS

First factorial experiment: variation of amounts of K_2HPO_4, $MgSO_4$ *and* KCl *added*

The preliminary experiments provided the range of variations on medium A which may be used as a basis of the factorial experiments. Talley & Blank (1941) used KCl in their medium and it was used here. The medium of the first factorial experiment concerned the salts K_2HPO_4, $MgSO_4.7H_2O$, and KCl. The KCl was present either at 0·002 M, or not added; chloride was always present in minute quantities in the Analar chemicals used. The K_2HPO_4 was used at 0·004, 0·008 and 0·012 M, while the $MgSO_4.7H_2O$ was at 0·0015, 0·003 and 0·0045 M. Thus eighteen possible solutions were set up each in five replicates, giving a total of 90 cultures in any one experiment. All cultures had an initial pH value suitable for good growth of this fungus. As growth proceeded the acidity uniformly increased to *c.* pH 2·0, when growth ceased. The results of this experiment are shown in Table 1 which includes the analysis of variance.

Hence, increasing the concentration of K_2HPO_4 from 0·004 M to 0·012 M markedly increased the dry weight of mycelium, whatever the concentration of $MgSO_4$ or KCl. The KCl level had no effect on dry weight. Increase of the $MgSO_4$ concentration from 0·0015 M to 0·0045 M in some cases increased dry weight yield slightly but, on the whole, not to a statistically significant extent. There was no evidence that the level of one salt affected the response to others.

Table 1. *Dry weight of mycelium and growth responses of* Rhizopus stolonifer *after 6–7 days growth at 26° on media with different concentrations of certain components*

Concentrations of K_2HPO_4: P_1, P_2, P_3 = 0·004 M, 0·008 M, 0·012 M; of $MgSO_4$: Mg_1, Mg_2, Mg_3 = 0·0015 M, 0·003 M, 0·0045 M; of KCl: 0·002 M or none. Difference required between weights for odds of 99:1 = 11·32 mg. for K_2HPO_4 and $MgSO_4$ and 9·02 mg. for KCl. Results given as mg. mean dry wt./mycelium.

	No KCl					0·002 M-KCl			
	Mg_1	Mg_2	Mg_3	Mean		Mg_1	Mg_2	Mg_3	Mean
P_1	92	103	112	106	P_1	108	106	105	103
P_2	124	130	139	131	P_2	113	121	137	120
P_3	162	148	148	153	P_3	142	160	156	153
Mean	126	127	133		Mean	121	129	133	

General mean = 127·6 mg. dry wt./flask.

General mean = 126·5 mg. dry wt./flask.

Analysis of variance

Required '*F*' and '*t*' values taken from Snedecor's tables (1934).

				F.	Required odds	
Variance	D.F.	Sum of Squares	Mean Square	Found	99:1	19:1
Total	89	57,275·66	643·55	—	—	—
K_2HPO_4	2	34,966·43	17,483·22	69·14	4·88	3·11
$MgSO_4$	2	1,298·02	649·01	2·57	4·88	3·11
KCl	1	31·21	31·21	—	—	—
$K_2HPO_4 \times MgSO_4$	4	1,098·31	274·58	3·99	4·88	3·11
$K_2HPO_4 \times KCl$	2	502·49	251·25	—	—	—
$MgSO_4 \times KCl$	2	192·96	96·48	—	—	—
2nd order interaction error	76	19,217·45	252·86	—	—	—

Second factorial experiment: variation of amounts of K_2HPO_4 *and* $(NH_4)_2SO_4$ *added*

This experiment was complementary to the first factorial experiment and sought to determine the relation between K_2HPO_4 and $(NH_4)_2SO_4$ in the medium *A*. The concentrations were: K_2HPO_4, 0·008, 0·012 and 0·016 M; and $(NH_4)_2SO_4$, 0·0125, 0·025 and 0·0375 M. The concentration of $MgSO_4$ was constant at 0·003 M; KCl was omitted. The other constituents of the medium and the conditions of growth were as in the previous experiment. The results are given in Table 2. It was again noticed that in each culture a favourable initial pH value between 7·0 and 8·0 fell to *c.* pH 2·0 when growth ceased.

The salts $(NH_4)_2SO_4$ and K_2HPO_4 taken singly show no significant effect except K_2HPO_4 at the 19:1 level only. The interaction of these salts, however, produced a highly significant effect at both probability levels, and hence a balance existed between them. Table 2 shows that no growth benefit was obtained by increasing the concentrations of $(NH_4)_2SO_4$ at the P_1 level, while at the P_2 level a very large increase in dry weight was noticed with increasing nitrogen concentration. At the P_3 level a similar increase in growth was obtained, but there was no significant difference between these two levels themselves. At the point of maximum dry weight of mycelium, i.e. when growth has ceased (somewhere between P_2 and P_3), chemical analysis showed that nitrogen was still present in the medium; thus $(NH_4)_2SO_4$ was not then limiting further growth. In both factorial experiments growth ceased when the pH value fell to *c.* 2·0. In the first experiment a balance was not found to exist between the salt constituents of the medium, but growth increased steadily with increasing concentration of phosphate hence the K_2HPO_4 acted as an essential nutrient. In the second factorial experiment a balance between K_2HPO_4 and $(NH_4)_2SO_4$ was found to exist. The writers have already shown (1956) that a suitably buffered medium containing 0·025 M-$(NH_4)_2SO_4$ will

Table 2. *Dry weight of mycelium and growth responses of* Rhizopus stolonifer *after 7 days growth at 26° in media with varying concentrations of* K_2HPO_4 *and* $(NH_4)_2SO_4$

Concentrations of K_2HPO_4: P_1, P_2, P_3 = 0·008 M, 0·012 M, 0·016 M; of $(NH_4)_2SO_4$: N_1, N_2, N_3 = 0·125 M, 0·025 M, 0·0375 M. Difference required between weights for odds of 99:1 = 13·22 mg. Results given as mg. mean dry wt./mycelium.

	N_1	N_2	N_3	Mean
P_1	123	126	123	124
P_2	139	159	177	158
P_3	130	197	171	166
Mean	131	161	157	—

General mean = 149·5 mg. dry wt./flask.

Analysis of variance

Required '*F*' and '*t*' values from Snedecor's tables (1934).

				F. Required odds		
Variance	D.F.	Sum of squares	Mean squares	Found	99:1	19:1
Total	44	41,082·8	933·7	—	—	—
K_2HPO_4	2	15,241·73	7620·87	4·24	4·29	3·13
$(NH_4)_2SO_4$	2	8,284·13	4142·07	2·30	4·92	3·13
$K_2HPO_4 \times (NH_4)_2SO_4$	4	7,184·54	1796·14	6·24*	3·60*	2·50*
Error	36	10,372·4	288·12	—		

* Note: where only one first-order interaction is significant and it is of greater magnitude than the error, then the mean square for the interaction is used in the determination of '*F*' values in place of the error.

produce up to about 300 mg. dry-weight mycelium and then the $(NH_4)_2SO_4$ begins to exert a limiting effect. It is possible then that in this experiment K_2HPO_4 also acted as a buffer. Thus buffering capacity of media becomes of importance in growth.

Variation of amounts of $(NH_4)_2SO_4$ and glucose added

In the preceding experiments the media contained 4% (w/v) glucose which gave a satisfactory growth and ensured that this substance did not limit growth by becoming exhausted in the medium. The experiment below describes the effect of varying the amount of glucose in the medium. Two sets of media were made up each containing 0·012 M-K_2HPO_4; 0·003 M-$MgSO_4.7H_2O$; trace metals 2 p.p.m. and glucose at 1, 2, 4, or 8 %; and either 0·0125 or 0·025 M-$(NH_4)_2SO_4$. Thus there were twenty-four different solutions with four concentrations of glucose and two concentrations of $(NH_4)_2SO_4$. The cultures were incubated as previously described. Four flasks of each variation of medium were withdrawn after 2 days growth, four more after 5 days and the remainder after 7 days growth. Thus it was possible to assess rate of growth as well as dry weight of the mycelium. The results are shown in Table 3.

Table 3. *Growth responses of* Rhizopus stolonifer *to variations in concentrations of glucose and* $(NH_4)_2SO_4$

Concentrations of $(NH_4)_2SO_4$: $N_1 = 0·0125$ M, $N_2 = 0·025$ M. Results given as mg. dry wt./mycelium. Initial pH: $N_1 = 7·25$, $N_2 = 7·42$.

Time in days	Percentage concentration glucose	Variation of final pH	Mean wt. in N_1	Mean wt. in N_2
2	1	6·42–6·88	13	12
	2	6·27–6·5	26	34
	4	5·9 –6·5	40	38
	8	5·88–6·3	63	71
5	1	2·38–2·51	91	100
	2	2·03–2·18	141	143
	4	2·01–2·19	150	169
	8	1·98–2·18	183	190
7	1	2·56–2·76	72	89
	2	2·1 –2·19	127	143
	4	2·05–2·17	132	161
	8	2·0 –2·17	171	199

At all periods after 2 days an increase of glucose concentration from 1 to 8 % showed a steady increase in mycelial weight. In each time period the greatest weight was obtained with 8 % glucose. At both concentrations of $(NH_4)_2SO_4$ an increase of glucose concentration greater than 1 % brought about a decrease in the length of the lag period, although after the second day the growth rates are all comparable. With a solution containing 0·025 M-$(NH_4)_2SO_4$ there was only a slight increase in weight compared with the solution at the 0·0125 M level at concentrations of 1, 2 and 8 % glucose; but at 4 % glucose after 5 and 7 days growth 0·025 M-$(NH_4)_2SO_4$ produced significantly higher weights of mycelium.

In this experiment the grand period of growth extended from after the second to the fifth day when the pH value fell to about 2·0, and in general the highest weights of mycelium were obtained on this day and thereafter there was a small drop. Growth ceases when the pH value reaches about 2·0 but usually this did not occur until the 6th to 7th day. During all periods the cultures with the 1 % glucose concentration showed an appreciably higher pH compared with the 2, 4 and 8 % levels of glucose.

Rate of growth

In this nutritional study the growth criterion has been dry weight of mycelium and the growth period was fixed at 6–7 days. The following experiment showed the effect of two other growth criteria, namely length of lag period and rate of growth. Two media were used for this purpose : (*a*) one of the optimal solutions from the second factorial experiment containing 0·012 M-K_2HPO_4; 0·003 M-$MgSO_4.7H_2O$; 0·025 M-$(NH_4)_2SO_4$; 4 % (w/v) glucose; and Zn, Fe, Mn at 2 p.p.m.; and (*b*) a solution which gave a minimal response containing 0·004 M-K_2HPO_4 and 0·0125 M-$(NH_4)_2SO_4$ with the other ingredients as above. Thirty-five flasks of each medium were set up and incubated as usual. After the first day's growth five flasks of each culture were removed each day for 8 days. The initial and final pH values were recorded and the results expressed as dry wt./mycelium. This experiment was carried out twice and the results are shown in Fig. 1.

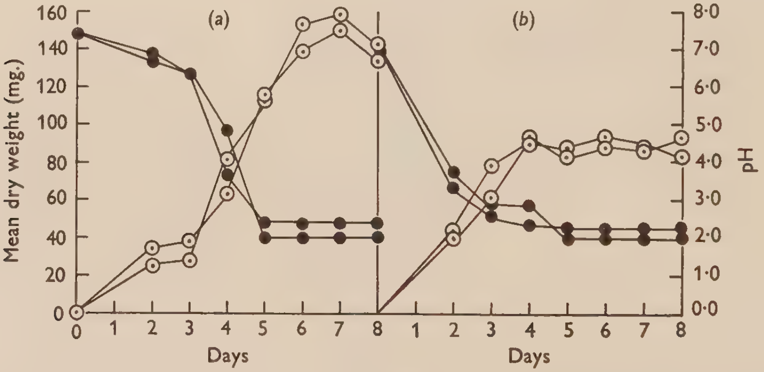

Fig. 1. Rate of growth and pH changes of *Rhizopus stolonifer* growing in optimal (*a*) and minimal (*b*) media (two experiments). ●—●, pH; ⊙—⊙, dry weight.

The optimal medium showed a 3-day lag period which coincided with germination, initial uptake of nutrients and the primary assimilation period. After the third day the grand period of growth set in rapidly. This rapid acceleration of growth was accompanied by a rapid drop in pH which probably coincided with the differential uptake of NH_4^+ ions in preference to SO_4^{--} ions in the medium (see Fothergill & Yeoman, 1956). Maximum growth occurred between pH values 6·4 and about 2·0, although a preliminary experiment showed that an initial pH value of 6·4 ultimately gave a decrease in dry weight of the mycelium. In the optimal medium the grand period of growth was followed by a drop in dry weight. On the other hand, the minimal medium showed a much shorter lag period of about 36 hr. The maximum dry weight was attained by the 4th day and it then persisted at a constant level throughout the remainder of the experimental period. The decline in dry weight had not set in with this medium even by the 8th day. The maximum rate of growth with this medium occurred after the pH values had fallen to between 3·0 and 2·0. Thus in the optimal solution the growth followed a typical pattern giving a normal sigmoid growth curve such as we expect in a normally metabolizing organism. In the minimal medium, however, growth became constant at a relatively early stage and with a low mycelial production. It is probable that in this medium the nutrients K_2HPO_4 or $(NH_4)_2SO_4$ were exerting a limiting effect on growth.

The importance of a balanced solution

It has already been shown in the first factorial experiment that a balance between the mineral salts was not required for good growth when $(NH_4)_2SO_4$ was at a constant concentration in the medium. In the third factorial experiment described below K_2HPO_4 and $MgSO_4$ were varied in a manner which would maintain the same balance between them in all solutions but which altered the ratio between the salts in different sets of media. Four treatments were given and in each the balance of these two salts was in the ratios 0·5: 1·0:2·0:4·0, when unity was represented by 0·012 M-K_2HPO_4 and 0·003 M-$MgSO_4.7H_2O$. Each of these four treatments was tested with $(NH_4)_2SO_4$ at concentrations of 0·0125, 0·025 and 0·05 M. The other ingredients in the media and the conditions of experiment were as before. The results were expressed as mg. dry wt./mycelium and they are shown in Table 4*A* and *B* which also includes the calculation for '*t*' determining the significance of the deviation between the mean dry weights.

These results indicated that treatments 3 and 2, 4 and 3, and 12 and 11 were not significantly different, while the others were different. It seems clear that the lower levels of all the salts produced a lower dry weight of mycelium up to treatment 6. After this treatment, even with the highest concentration of $(NH_4)_2SO_4$, the lower concentrations of K_2HPO_4 and $MgSO_4$ in treatments 9 and 10 still produced a low dry weight of mycelium. This experiment confirmed the previous conclusion that there is no balance between K_2HPO_4 and $MgSO_4$, but it also indicated that a balance of these salts with $(NH_4)_2SO_4$ was necessary for good growth. The significant effect of this balance became evident from the mean growth responses and interactions in Table 4*B*. At the 0·5 and 1·0 salt ratios the buffer capacity of the phosphate must have been limiting growth because increasing the concentration of $(NH_4)_2SO_4$ had only little effect upon the mycelial yields. By increasing the $(NH_4)_2SO_4$ concentrations at the 2·0 salt ratios an increase in dry weight was brought about; at this ratio buffer capacity probably became limiting only at the highest concentration of $(NH_4)_2SO_4$. Finally, with the 4·0 salt ratio an increase in the nitrogen concentration from 0·0125 M to 0·025 M gave a highly significant effect and produced the best mycelial growth of all the experiments. By further raising the nitrogen concentration to 0·05 M no further significant effect was produced. In this case the acidity concomitant with the higher nitrogen concentration was limiting growth. Thus it is apparent that no significant increase of dry weight will result in media such as these by increasing the buffer capacity while the nitrogen salt is limiting, or by raising the concentration of $(NH_4)_2SO_4$ while the buffer capacity is limiting. It may be concluded that the importance of the balance existing between the combined mineral salts and $(NH_4)_2SO_4$ lies in the buffering capacity of the mineral salts. As $MgSO_4$ is not a buffering agent the true balance in this medium is between K_2HPO_4 and $(NH_4)_2SO_4$. If then

Table 4. *Dry weight of mycelia (A) and mean growth responses (B) of* Rhizopus stolonifer *to different ratios of* K_2HPO_4 *and* $MgSO_4$ *with the same balance and different concentrations of* $(NH_4)_2SO_4$

Molar concentrations of K_2HPO_4: P_1, P_2, P_3, P_4 = 0·006, 0·012, 0·024, 0·048; of $MgSO_4$: Mg_1, Mg_2, Mg_3, Mg_4 = 0·0015, 0·003, 0·006, 0·012; of $(NH_4)_2SO_4$: N_1, N_2, N_3 = 0·0125, 0·025 0·05. Initial pH = 7·62–6·85. Value of '*t*' required at the 99:1 level for differences to be significant is 2·447. Results given as mg. mean dry wt./mycelium.

A. *Dry wt. mycelia*

Treatment soln. no.	Salts			Range of final pH	Mean wt. mycelium	Differences	'*t*'
	P	Mg	N				
1	P_1	Mg_1	N_1	2·06–2·1	100	—	—
2	P_2	Mg_2	N_1	2·1 –2·2	157	57	5·451
3	P_3	Mg_3	N_1	2·85–3·1	142	15	1·511
4	P_4	Mg_4	N_1	3·44–3·68	151	9	1·697
5	P_1	Mg_1	N_2	2·13–2·23	110	41	4·941
6	P_2	Mg_2	N_2	2·13–2·2	141	31	2·766
7	P_3	Mg_3	N_2	2·19–2·36	198	57	4·773
8	P_4	Mg_4	N_2	2·96–3·0	240	42	3·027
9	P_1	Mg_1	N_3	2·2 –2·28	122	118	10·26
10	P_2	Mg_2	N_3	2·20–2·23	140	18	2·534
11	P_3	Mg_3	N_3	2·15–2·32	232	92	4·843
12	P_4	Mg_4	N_3	2·85–2·94	235	3	<1

B. *Mean growth response and interactions*

Salts = K_2HPO_4 and $MgSO_4$; figures in brackets = treatment nos.

$(NH_4)_2SO_4$ concn.	Mean wt. mycelium
N_1	138
N_2	172
N_3	182

Salt ratio concn.	Mean wt. mycelium
0·5	111
1·0	146
2·0	191
4·0	209

Interaction salt ratio × $(NH_4)_2SO_4$ mg.		
0·5 × N_1	100	(1)
× N_2	110	(5)
× N_3	122	(9)
1·0 × N_1	157	(2)
× N_2	141	(6)
× N_3	140	(10)
2·0 × N_1	142	(3)
× N_2	198	(7)
× N_3	232	(11)
4·0 × N_1	151	(4)
× N_2	240	(8)
× N_3	235	(12)

the acidity of the medium plays such a large part in determining the amount of mycelial growth, by using better buffering agents than K_2HPO_4 mycelial growth should be greatly increased. In fact, as already mentioned, the writers (1956) have shown elsewhere that by using anhydrous sodium succinate, which does not act as a significant nutrient for this fungus in this medium but which is an excellent buffer, mean dry mycelial weights of nearly 300 mg. have been obtained. When K_2HPO_4 is used as a nutrient and as a buffer the highest yield of mycelium is obtained when the fungus is grown for 6–7 days at 26° in a liquid medium containing: 4% (w/v) glucose; $0{\cdot}048\,\text{M-}K_2HPO_4$; $0{\cdot}012\,\text{M-}MgSO_4.7H_2O$; $0{\cdot}025\,\text{M-}(NH_4)_2SO_4$; and Zn, Fe, Mn at 2 p.p.m.

Morton & Macmillan (1954) carried out an extensive study of the assimilation of nitrogen from ammonium salts and nitrate by several fungi in which they showed that, in the presence of ammonium salts, various organic acids act as buffers. The chief fungus they worked with was *Scopulariopsis brevicaulis*, but this is a fungus which uses KNO_3 alone, unlike Rhizopus. Their results, however, are not strictly comparable with those presented here for the above reason and also because they did not analyse the effect of KH_2PO_4, present in their basal medium, on the assimilation of ammonium. Nevertheless, these experiments confirm those of Morton and Macmillan by showing that assimilation of ammonium is greatly increased in the presence of K_2HPO_4 acting as a buffer. The writers (1956) have already shown by chromatographic analysis that in a medium containing $(NH_4)_2SO_4$, sulphuric acid forms in the medium and the phosphate buffers this substance. Inert buffers, like 'Sigma' buffer (2-amino-2-hydroxymethylpropane—1:3-diol) act better than phosphate, and acetate and succinate act best of all. It seems in fact if all these results are considered that growth is proportional to buffer capacity and the latter may become an important growth-limiting factor.

The authors would like to thank their colleague Mr H. Campbell, B.A., F.S.S., for his kind help with the statistics.

REFERENCES

Fothergill, P. G. & Ashcroft, R. (1955). The nutritional requirements of *Venturia inaequalis*. *J. gen. Microbiol.* **12**, 387.

Fothergill, P. G. & Raine, L. C. D. P. (1954). The mineral nutritional requirements of *Mucor hiemalis* Wehm. *J. gen. Microbiol.* **10**, 17.

Fothergill, P. G. & Yeoman, M. M. (1956). Acidity and growth of *Rhizopus stolonifer* in mineral culture media. *Bull. Jard. bot. Brux.* **26**, 371.

Morton, A. G. & Macmillan, A. (1954). The assimilation of nitrogen from ammonium salts and nitrate by fungi. *J. exp. Bot.* **5**, 232.

Robbins, W. J. (1924). Isoelectric points for the mycelium of fungi. *J. gen. Physiol.* **6**, 259.

Robbins, W. J. (1937). The assimilation by plants of various forms of nitrogen. *Amer. J. Bot.* **24**, 243.

Snedecor, G. W. (1934). Calculation and interpretation of analysis of variance and co-variance. *Iowa St. Coll. Div. Indust. Sci. Monogr.* **1**. Ames, Iowa.

Talley, P. J. & Blank, R. L. M. (1941). A critical study of the nutritional requirements of *Phymatotrichum omnivorum*. *Plant Physiol.* **16**, 1.

(*Received* 17 *May* 1957)

Brown, A. D. (1957). *J. gen. Microbiol.* 17, 640–648.

Some General Properties of a Psychrophilic Pseudomonad: the Effects of Temperature on some of these Properties and the Utilization of Glucose by this Organism and *Pseudomonas aeruginosa*

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SUMMARY: A psychrophilic species of the genus *Pseudomonas* was found to be capable of growth in a simple defined medium with any one of a number of carbon sources. The growth requirements were the same at 0° and 20°. The organism vigorously oxidized glucose to gluconic and 2-ketogluconic acids. Rates of O_2 uptake were measured over the range 0–40° and compared with similar measurements on *P. aeruginosa*. Under the experimental conditions values of Q_{O_2} for the psychrophile were higher than those for *P. aeruginosa* at all temperatures, including those above 30° at which temperatures the psychrophilic organism does not grow. The values of Q_{O_2} and their temperature coefficients were dependent on conditions of cultivation. It is concluded that the systems involved in the oxidation of glucose and gluconic acid by the two organisms may not be greatly different in those physical properties which determine their temperature relations.

Some types of bacteria which flourish under certain physical extremes, e.g. low water activities (the halophils) and high temperatures (the thermophiles) have been moderately well studied. Very little information about the physiology of the low-temperature bacteria (the psychrophils) is available, however. It is the purpose of the present report to describe some experiments undertaken to determine some general properties of a typical psychrophil, to compare some of these properties with those of a closely related mesophilic species and to suggest ways in which it might be possible to study the special properties of the psychrophil which enable it to thrive at low temperatures.

METHODS

Media. The following growth media were used:

Medium 1. 'General use' medium (Stephenson, 1949). An inorganic salts medium of concentration 0·057 M and pH 6·9, carbon source added as indicated.

Medium 2. 'Inorganic salts' medium of the following composition: $(NH_4)_2SO_4$, 0·005 M; $MgSO_4$, 0·005 M; Na_2HPO_4, 0·0126 M; KH_2PO_4, 0·0066 M; NaCl, 0·019 M; KCl, 0·0114 M; Na_2SO_4, 0·0076 M; $FeSO_4$, 0·0001 M. The final pH value was 7·0; carbon source added as indicated.

Medium 3. Hartley's tryptic digest broth (TDB).

Medium 4. 'Difco' Nutrient Broth (NB).

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Modified Sørensen buffers containing $MgSO_4$ were prepared to a molarity of 0·057 in the pH range 6·1–7·0. Solutions of sugars and other organic compounds for use in these various media were prepared in 10 % (w/v) concentration and sterilized by filtration.

Organisms. A psychrophilic bacterium and a strain of *Pseudomonas aeruginosa* were used. The psychrophilic organism was isolated from chilled beef and is held in the stock culture collection of the Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W., Australia, under the identification number 1482. Stock cultures were maintained on nutrient agar at about 0° with subsidiary cultures in TDB or NB.

For all purposes inocula were prepared by incubating the psychrophile in 6 ml. tubes of TDB or NB for 3 days at 20° and *Pseudomonas aeruginosa* in 6 ml. tubes of NB overnight at 37°. Washed inocula were used in growth rate studies.

For growth rate studies 80 ml. of medium 1, or 60 ml. of medium 2 were dispensed in conical flasks of 200 ml. capacity, and agitated by a stream of sterile air. Samples were withdrawn through a side arm under pressure of the air stream. Growth rates were followed by colony counts or turbidimetrically. In the latter method turbidity was calibrated against the wet weight of cells.

Suspensions for manometric experiments were prepared by growing the organisms to their early stationary phase in Difco NB (1 l.) containing D-glucose (0·2 %). The medium was seeded with 18 ml. of inoculum and incubated with occasional manual shaking. *Pseudomonas aeruginosa* was harvested after 25 hr. at 37° or 69 hr. at 20°. The psychrophil was harvested after 69 hr. at 20°. The organisms were washed at 20° in saline (NaCl, 0·3 %, w/v) and phosphate buffer (0·057 M, pH 7) and finally resuspended in water (10 ml.) at 0–5° for 30 min.

Manometry. Rates of O_2 uptake were determined in duplicate flasks by conventional Warburg techniques at the stated temperatures.

Analysis of cultures. For the analysis of reaction products, the organisms were grown in the aerating flasks in medium 1 containing D-glucose (1 %, w/v) or washed suspensions were incubated under the same conditions in the phosphate buffer (pH 7) containing D-glucose (1 %). After centrifugation, cations were removed from the supernatant fluid by passage over the resin Amberlite IR–120. The effluent was neutralized to phenolphthalein with NH_4OH and evaporated at *c.* 40° under vacuum to 0·2–0·5 ml. Gluconic acid was identified first by paper chromatography at 20° with the following solvent mixtures: *n*-butanol + acetic acid + water (6 + 1 + 2); *n*-propanol + ammonia solution sp.gr. 0·880 (6 + 4); water-saturated phenol. Its identity was confirmed by elution from the paper chromatogram and oxidation with Fe^{+++} ions and H_2O_2 (after Hockett & Hudson, 1934) to a substance which was chromatographically indistinguishable from D-arabinose. 2-Ketogluconic acid was identified by its chromatographic behaviour in the above solvents, its reactions with ammoniacal silver nitrate (reduction), aniline phthalate (the pink of aldopentoses), its specific colour reaction with *O*-phenylenediamine (Lanning & Cohen, 1951) and by the chromatography of its 2:4-dinitro-phenylhydrazone in the butanol+acetic acid and propanol +ammonia solvent mixtures.

RESULTS

General properties of the psychrophil

The psychrophilic bacterium was a non-pigmented, monotrichous *Pseudomonas* sp. representative of the organisms to be described elsewhere by Brown & Weidemann. It was capable of growth at 0° and 20° in medium 1 with any one of a number of simple carbon sources (Table 1). Growth did not occur at 37° in either defined medium or Difco NB unless a trace (1 loopful) of the more complex TDB were added. Under such conditions or in TDB itself, restricted growth occurred.

Table 1. *Ability of the psychrophilic pseudomonad to utilize various substances for growth*

The numerals in parentheses refer to the number of days of incubation at 20° for the appearance of definite turbidity in Medium 1 + carbon source at 1 % (w/v) concentration. Growth rates at 20°, determined by viable counts, were of similar magnitude with glucose, gluconate or succinate as carbon source.

D-Arabinose	+ (1–2)	Citrate	+ (1–2)
D-Glucose	+ (1–2)	Fumarate	+ (1–2)
Glycerol	+ (2–3)	Gluconate	+ (1–2)
Lactose	–	α-Ketoglutarate	+ (1–2)
Maltose	–	Succinate	+ (1–2)
Sucrose	–		
D-Xylose	+ (2–3)		

Sodium malonate (0·01 M) had no effect on growth in medium 1 with any of the possible carbon sources. Sodium iodoacetate (0·001 M) greatly inhibited growth and at 0·01 M completely suppressed it. The rate of acid production from glucose was not affected by iodoacetate at either concentration.

Quantitative observations

Although qualitative observations had shown no absolute differences in nutrient requirements at 20° and 0° it was conceivable that there might be quantitative differences in relative growth rates and durations of the lag phase in complex and defined media. Table 2 summarizes results in which glycerol and D-glucose were used as separate carbon sources in basal medium 1. It is evident that lowering the temperature from 20° to 0° affected growth rates in TDB and the glycerol medium about equally and somewhat less than in the glucose medium. The effect of the temperature change on the duration of the lag phase was less in defined medium no. 1 with both carbon sources than it was in the broth. Fig. 1 summarizes the effect of temperature over the range 0–40° in a different medium (medium 2 + 1 % (w/v) glucose) on lag and growth rate of the psychrophil and *Pseudomonas aeruginosa*.

Table 2. *Rates of growth and durations of the lag phase of the psychrophilic pseudomonad at two temperatures in complex TDB medium and in defined media*

Medium	T.D.B.	Defined medium no. 1+glycerol (1 %, w/v)	Defined medium no. 1+glucose (1 and 2 %, w/v)*
20° lag† (hr. ± 10 %)	3	14	13·5
Growth rate (gen./hr.)	0·75	0·50	0·83
0° lag† (hr. ± 10 %)	55	103	127
Growth rate (gen./hr.)	0·078	0·061	0·060
Ratio, lag 0°:20°	18	7	9
Growth rate 20°:0°	9·6	8·2	13·8

* Variations of this magnitude in glucose concentration had no effect on either lag or growth rate.

† Defined as the length of the straight line drawn parallel to the time axis through the logarithm of the population at time zero to the point of intersection with the straight line representing the logarithmic growth phase. Growth was followed by viable counts.

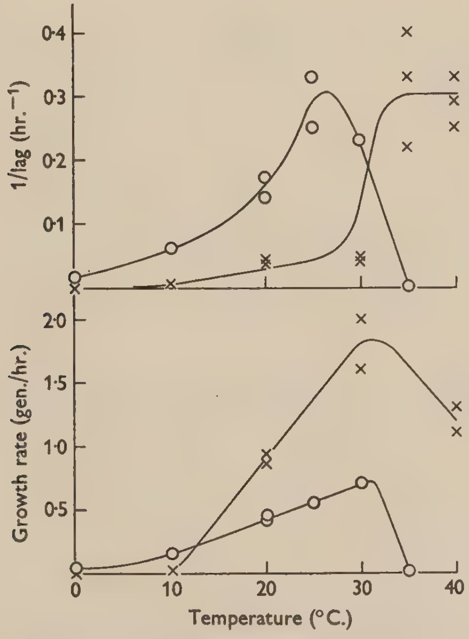

Fig. 1. The effects of temperature on the duration of the lag phase (upper figure) and on growth rate (lower figure) of *Pseudomonas aeruginosa* (×) and the psychrophil (○). Growth was followed turbidimetrically in medium 2 containing glucose (1 %). Inocula were the contents of 6 ml. culture in 'Difco' Nutrient broth centrifuged and resuspended in water (1 ml.). (Note that absolute values of growth rate of the psychrophil were less in this medium than for example, in medium 1, see Table 2.)

Observations on glucose utilization

The production of acid. At 20°, under conditions of relatively vigorous aeration either during growth in defined medium or during incubation of a washed suspension in a buffer, the psychrophil produced acid from excess glucose (1 %) until an acid concentration equivalent to pH 3·0–3·2 was obtained. At this stage growth and further breakdown of the acids so produced ceased. At 0° the lowest pH values recorded under similar conditions were of the order of 4. With lower initial concentrations of glucose (e.g. 0·5 %) such low pH values were not reached, and after the exhaustion of the glucose supply the acidic reaction products were utilized, accompanied by an increase of pH value of the medium and further growth. Under similar conditions, but at 37°, the lowest pH value reached with *Pseudomonas aeruginosa* was 4·8–4·9. At 0° no change in the pH value of medium 1 or the buffer was detected after a month's incubation of *P. aeruginosa* in the presence of glucose. During this time the suspension became sterile but the dead organisms retained their ability to oxidize glucose to acid on rewarming to 37°. The identity of these acids was the same as produced by viable organisms (see next section).

The rates of acid production from glucose by a washed suspension of the psychrophile in phosphate buffer (pH 7) were: at 20°, $0{\cdot}96 \times 10^{-5}$ g. equiv. H^+/mg. dry wt. organism/hr.; at 0°, $1{\cdot}07 \times 10^{-6}$ g. equiv. H^+/mg. dry wt. organism/hr., the temperature coefficient (ratio 20°:0°) being 9·0. A degree of lysis (28–48 %) occurred at both temperatures shortly after the preparation of the suspension in the buffer. The above rates were expressed on the initial weight of organism in the buffer, i.e. before lysis. When rates are expressed on weight of whole organisms after lysis had ceased the corresponding temperature coefficient is 7·5.

The identity of the acids formed. The only substances found to accumulate in the medium from oxidation of glucose at 20° and 0° by the psychrophil were gluconic and 2-ketogluconic acids. Volatile acids were not specifically sought and the conditions of cultivation and analysis were such that very volatile acids, e.g. formic and acetic acids, would have escaped detection. No organic phosphates were detected on chromatograms with the molybdate reagent described by Hanes & Isherwood (1949). In the presence of iodoacetate (0·005 M) a substance which behaved chromatographically as glycollic acid also accumulated.

Many studies with the mesophilic species of *Pseudomonas* have established the accumulation of gluconic and 2-ketogluconic acids (see reviews by Gunsalus, Horecker & Wood, 1955; Wood, 1955). It was confirmed that the strain of *P. aeruginosa* used in the present work also accumulated these substances at 20° and 37° but, as would be expected from the foregoing measurements of pH value, in lower concentration than produced by the psychrophil. (As no attempt was made to neutralize the acids as they were formed this difference is probably merely an indication of differences in acid tolerance.)

Manometric measurements. Preliminary observations were made at 30° with *Pseudomonas aeruginosa* and at 25° with the psychrophil. Under these conditions whole organisms of *P. aeruginosa* oxidized glucose and gluconate with total uptakes of 5 and 4·5 mole O_2/mole glucose and gluconate, respectively. The psychrophil used 4·5 and 4 mole O_2/mole glucose and gluconate, respectively. The effect of pH value on rate of O_2 uptake was determined in the range pH 6·1–7·0. *P. aeruginosa* oxidized glucose at the same rate throughout this range but oxidized gluconate fastest at pH 6·1. The mean and total range of rates of two or three measurements of Q_{O_2} with this substrate at these limits of pH were Q_{O_2}, 83 ± 9 and 72 ± 7, at pH 6·1 and 7 respectively. The psychrophil also oxidized gluconate fastest at pH 6·1 (Q_{O_2} 178 ± 25 and 157 ± 18 at pH 6·1 and 7, respectively), but glucose was oxidized most rapidly at pH 7·0 (see Fig. 2).

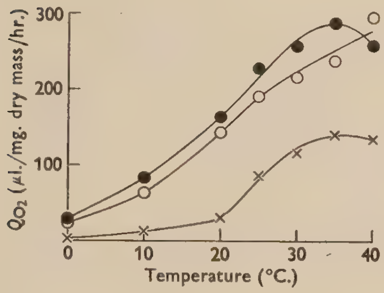

Fig. 2

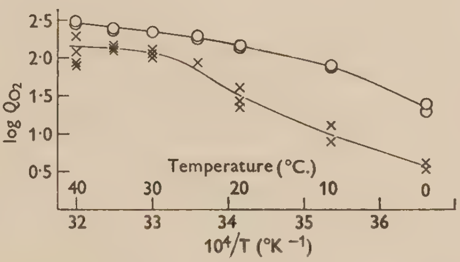

Fig. 3

Fig. 2. The effect of temperature on Q_{O_2} in the presence of glucose for the psychrophil at pH 7 (●) and pH 6·1 (○) and for *Pseudomonas aeruginosa* (×) at pH 6·1. The respiration flask contained phosphate buffer (1·0 ml.), cell suspension (1·53 mg. dry mass/ml.; 1·7 ml.), substrate (0·556 M; 0·3 ml.) with KOH in the centre cup. Endogenous respiration has been subtracted. The points represent means of the replicates shown in Fig. 3. Similar results, but with lower overall values of Q_{O_2}, were obtained when sodium gluconate was the substrate.

Fig. 3. Log Q_{O_2} with glucose at pH 6·1 v. the reciprocal of the absolute temperature (T) for the psychrophil (○) and for *Pseudomonas aeruginosa* (×). Corresponding curves for sodium gluconate have a somewhat more obvious change in slope near 30° in the case of *P. aeruginosa* and lower overall values of log Q_{O_2}.

The effects of temperature on oxygen uptake rates were determined at pH 6·1 with additional measurements at pH 7 for oxidation of glucose by the psychrophil. The results of these measurements are shown in Fig. 2, from which it is apparent that under the experimental conditions the psychrophil was capable of utilizing the two substrates more vigorously than *Pseudomonas aeruginosa*. This greater activity was displayed at all temperatures including those above the maximum at which the psychrophil can grow. Reference to Fig. 3 also shows a different effect of temperature over the range 0–40° on the processes in the two organisms. These logarithmic curves show in the case of *P. aeruginosa* a relatively sudden change in slope near 30°. No corresponding changes in slope are apparent in the curves for the psychrophil.

The effect of growth temperature on Q_{O_2} *of* Pseudomonas aeruginosa. In order to assess the effect of growth temperature on ability to oxidize the two substrates, *P. aeruginosa* was grown for 69 hr. at 20° and the rates of O_2 uptake measured at 0° and 20°. The results are given in Table 3. It will be seen that growth at the lower temperature led to greater values of Q_{O_2} at both temperatures, a decreased temperature coefficient over this range and a changed pH relationship for the oxidation of glucose. Thus, when grown under identical conditions, the two organisms resembled each other more closely. *P. aeruginosa*, which had been grown in this way at 20° would not, however, grow at 0°.

Table 3. *The effect of growth temperature on rates of* O_2 *uptake by* Pseudomonas aeruginosa

Incubation temperature of suspension	*P. aeruginosa*					Psychrophilic pseudomonad. Grown at 20°		
	Grown at 37°		Grown at 20°					
	Glucose pH 6·1	Gluconate	Glucose		Gluconate	Glucose		Gluconate
			pH 6·1	pH 7·0		pH 6·1	pH 7·0	
	Q_{O_2} values							
30°	118	79	185	247	164	224	268	182
0°	3·8	1·7	8·3	12	11	22	29	20
Temperature coefficient (ratio 30°:0°)	31	46	22	21	15	10	9·2	9·1

DISCUSSION

It is evident that in respect of its growth requirements, its oxidative attack on glucose and its accumulation of reaction products, e.g. gluconic and 2-keto-gluconic acids, the psychrophilic bacterium is typical of the genus *Pseudomonas*. The ease of growth on D-arabinose (Table 1) and the probable accumulation of glycollic acid in the presence of iodoacetate might be related to the reported breakdown of D-arabinose by *P. saccharophila* to give pyruvic and glycollic acids (Doudoroff, De Ley, Palleroni & Weimberg, 1956). Thus nothing emerges from these observations which obviously distinguishes the psychrophil from other species which have been well studied to date, namely, *P. aeruginosa*, *P. fluorescens*, *P. saccharophila* and *P. hydrophila*.

Examination of the psychrophilic organism at 0°, however, did reveal some properties which might be characteristic of psychrophilic bacteria. Its simple growth requirements are not changed by lowering the temperature to 0°. Indeed, in respect of at least one property, namely the duration of the lag phase, it might be argued that 0° is a more favourable temperature for this organism than 20°. The different temperature coefficients of the lag in defined media (no. 1) and TDB (Table 2) have the effect of decreasing the proportional differences between the lag phases in the two kinds of medium when the temperature is lowered to 0°. For example, at 20° the lag in defined medium was about 4·5 times as great as in TDB, but at 0° this ratio was reduced to about 2.

The comparisons of the growth rates and lags of the psychrophil and *Pseudomonas aeruginosa* (Fig. 1) show differences which might be indicative of more than a simple species difference and of the ways in which the inocula for these experiments were prepared. Notwithstanding the large measure of uncertainty which frequently accompanies measurements of lag it is clear that under the conditions used here at least, the psychrophil was able to begin multiplication much sooner than *P. aeruginosa* over almost all of that part of the temperature range in which growth of both species occurred. This suggests that the psychrophil might be better able to adjust itself (no implications about the mechanism of adjustment are intended) to a new and potentially difficult environment than is *P. aeruginosa*.

As the suspensions used in the manometric experiments were grown under similar conditions of time and temperature to those used in the comparisons presented in Fig. 1 it is conceivable that the higher rates of glucose oxidation by the psychrophil are related to its shorter lag phase. The difference in final concentrations of acid produced by the two species might reflect differences in the importance of some metabolic pathways of the two organisms or, more likely, it might merely reflect differences in acid tolerance. Whether these three features, namely a comparatively short lag phase in synthetic media, vigorous oxidative ability over a relatively wide range of temperature and possibly greater tolerance than the mesophilic species to H^+ are generally characteristic of the psychrophilic species of *Pseudomonas* has yet to be ascertained.

Table 3, however, shows that quantitative differences between the two organisms are subject to modification by variations in the growth conditions of the cells. It is probably reasonable to conclude that under certain conditions of growth the relevant enzymes in the two species might not be greatly different in respect of those physical properties which determine their temperature relations. If this is true it is clear that an explanation of the different temperature minima for growth must be sought elsewhere than in the initial stages of glucose oxidation, possibly in metabolic differences or in physical differences in some essential enzyme systems. It is apparent, however, that useful information is to be obtained from a study of simple systems which need not necessarily be essential to the growth of the organism. In such a study it is obviously desirable to compare these systems in organisms which have been grown under carefully selected conditions of temperature and time and to obtain evidence of the relative contributions of cellular organization on the one hand and the physical properties of the relevant enzymes on the other to results such as have been obtained already and are likely to be obtained after different conditions of growth.

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REFERENCES

Doudoroff, M., de Ley, J., Palleroni, N. J. & Weimberg, R. (1956). New pathways in oxidation of D-galactose and of D-arabinose. *Fed. Proc.* **15**, 794.

Gunsalus, I. C., Horecker, B. L. & Wood, W. A. (1955). Pathways of carbohydrate metabolism in microorganisms. *Bact. Rev.* **19**, 79.

Hanes, C. S. & Isherwood, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

Hockett, R. C. & Hudson, C. S. (1934). Improvements in the preparation of D-arabinose from calcium gluconate. *J. Amer. chem. Soc.* **56**, 1632.

Lanning, M. C. & Cohen, S. S. (1951). The detection and estimation of 2-ketohexonic acids. *J. biol. Chem.* **189**, 109.

Stephenson, M. (1949). *Bacterial Metabolism.* 3rd ed. London: Longmans Green and Co.

Wood, W. A. (1955). Pathways of carbohydrate degradation in *Pseudomonas fluorescens*. *Bact. Rev.* **19**, 222.

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A Method for Isolating Streptomyces

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The isolation of Streptomyces from crowded or contaminated cultures is often a matter of some difficulty. We have devised a simple method which has been used with some success in the case of sporulating cultures. A small piece of medium bearing the organism to be isolated is cut out with a sterile spatula and placed on the bottom of the cotton wool plug of a tube containing a sterile nutrient agar slope. The medium will adhere to the plug, leaving the mycelium on the outward surface. The tube is then tapped gently, so that spores are scattered on the fresh medium. The tube should be held at a very slight angle to ensure even distribution; too low an angle will tend to confine the spores to the upper portion of the slope. The plug can now be transferred to a second tube and the operation repeated. By this means, a series of tubes bearing decreasing numbers of discrete colonies can be obtained; the last tube may be closed with the original plug. Fig. 1 shows such a series, the position of the inoculum on the plug being indicated by the arrow. In the case of cultures contaminated with bacteria a series of three tubes is usually adequate, since the bacteria remain on the plug, but in the case of fungi or mixed cultures of Streptomyces it is advisable to use a longer series. Apart from the problem of isolation, the method is valuable as a simple way of obtaining isolated colonies on an agar slope culture.

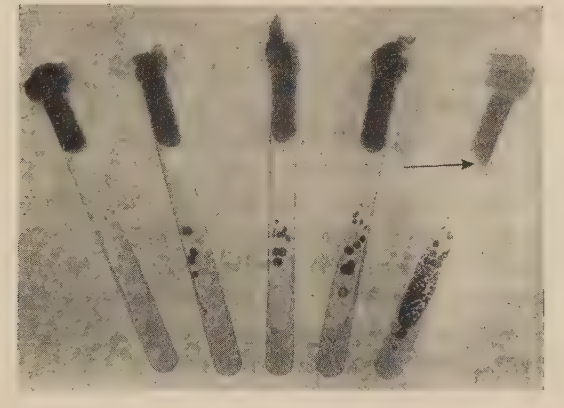

Fig. 1

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The Hexose Constituents of some Shigella Polysaccharide Hydrolysates

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SUMMARY: Polysaccharides obtained from twelve previously unextracted Shigella strains were hydrolysed and analysed chromatographically to determine their hexose constituents. The polysaccharides extracted from eight strains of the Flexner group (NCTC strains 3, 4833, 8205, 8206, 8201, 8207, 2245 and 4839), all contained the same hexose constituents, namely glucosamine, glucose and rhamnose. *Shigella shigae* (NCTC 8005) polysaccharide contained glucosamine, galactose and rhamnose. *S. sonnei* (NCTC 8220) polysaccharide contained glucosamine, galactose and glucose. *S. schmitzi* (NCTC 8218) polysaccharide contained glucosamine, galactose, glucose and rhamnose. *S. alkalescens* (NCTC 7925) polysaccharide contained glucosamine, galactosamine, glucose and galactose.

The introduction of carbohydrate paper chromatography by Partridge (1948*a*) provided a simple method of confirming the chemical studies of Shigella polysaccharides by Morgan (1931, 1932, 1936, 1937), Morgan & Partridge (1940, 1941) and Goebel, Binkley & Perlman (1945), and of extending these studies to other Shigella polysaccharides. Partridge (1948*b*) confirmed the presence of glucosamine, galactose and rhamnose in the hydrolysis products of *Shigella shigae* polysaccharide extracted by the diethyleneglycol method. Jesaitis & Goebel (1952) identified glucosamine, galactose and glucose in the hydrolysis products of *S. sonnei* polysaccharide extracted by the infusion of phenol-killed organisms with warm distilled water. Slein & Schnell (1952), and Slein (1952), identified glucosamine, glucose and rhamnose in the hydrolysis products of *S. flexneri* type 3 polysaccharide. Davies (1955) identified glucosamine, galactose, glucose and rhamnose in the hydrolysis products of *S. schmitzi* polysaccharide and galactosamine, glucosamine, galactose and glucose in *S. alkalescens* polysaccharide. The trichloroacetic acid method of extraction was used in the preparation of the last two polysaccharides. Aldoheptoses have been identified in a lipopolysaccharide extracted from *S. sonnei* by Jesaitis & Goebel (1952), from a phosphorus-containing polysaccharide extracted from *S. flexneri* type 3 by Slein & Schnell (1953), and in a lipopolysaccharide extracted from *S. shigae* by Davies, Morgan & Record (1955).

In the present work the trichloroacetic acid method of extraction (Boivin & Mesrobeanu, 1934, 1937) was used because it is now generally accepted that antigenic preparations can be obtained in this way from the majority of Gram-negative bacilli (Boivin & Mesrobeanu, 1934, 1937; MacKenzie, Pike & Swinney, 1940; Davies, 1955), although it fails to remove the analogous substances from *Pasteurella pestis* (Girard, 1941), and some *Escherichia coli* strains (MacPherson, 1956). When applicable the diethyleneglycol method

(Morgan, 1937) is the most satisfactory method as this solvent is effective at neutrality and at normal temperatures. Unfortunately it is not generally applicable to the Shigella group as the yield with some strains is poor and with *Shigella flexneri* type 6 no antigen can be recovered at all (Goebel, Binkley & Perlman, 1945). Trichloroacetic acid extracts are not, however, homogeneous but consist mainly of two components, the one being a polypeptide-phospholipid-polysaccharide complex or antigenic complex, and the other free serologically-active polysaccharide (Davies, Morgan & Mosimann, 1954). The yields of polypeptide-phospholipid-polysaccharide complex and of polysaccharide are not so high as with other methods of extraction but the preparations are less heavily contaminated with other cell constituents, e.g. phenol extracts contain a certain amount of simple protein (Morgan & Partridge, 1941) and pyridine extracts comparatively large amounts of nucleic acids (Goebel *et al.* 1945).

The object of the present work was to identify by chromatographic methods the hexose constituents of the polysaccharides from: (1) a representative group of Flexner strains, one strain being selected from each of the eight main types; and (2) four other members of the genus *Shigella*.

METHODS

The organisms used in this investigation were the following, the numbers in brackets being the NCTC numbers: *Shigella flexneri* type 1a (3); *S. flexneri* type 2a (4833); *S. flexneri* type 3 (8205); *S. flexneri* type 4a (8206); *S. flexneri* type 5 (8201); *S. flexneri* type 6 (8207); Flexner X (Andrewes & Inman; 2245); Flexner Y (Andrewes & Inman; 4839); *S. shigae* (8005); *S. sonnei* (8220); *S. schmitzi* (8218); *S. alkalescens* (7925).

Preparation of organisms. The purity of each culture used for mass inoculation was checked by morphological, biochemical and serological tests and its smoothness confirmed by colonial appearance and salt stability. The cultures were grown on ox-heart extract agar at 37° for 20 hr. and washed off in 0·9 % (w/v) saline. The organisms were washed a further three times in 0·9 % (w/v) saline and then twice in distilled water. The bacterial sludge obtained after the last wash was suspended in 30 ml. distilled water and the organisms killed by adding 10 vol. acetone at $-15°$. After two more washes in acetone the organisms were dried *in vacuo* over conc. H_2SO_4 and stored at 0°. Each Flexner mass culture, harvested from five hundred 4-in. Petri plates, yielded approximately 6·0 g. acetone-dried bacteria, while each mass culture of the other members of the genus, harvested from two hundred and fifty 4-in. Petri plates, yielded approximately 3·0 g. The bacteriological purity of each plate was checked by inspection and by slide agglutination with the appropriate standard antiserum obtained from the Standards Laboratory, Colindale.

Trichloroacetic acid extraction of bacteria. The acetone-dried bacteria were extracted for 4 hr. by the method of Boivin & Mesrobeanu (1934) with N-trichloroacetic acid. After separation of the bacteria by centrifugation the extracts were dialysed to remove trichloroacetic acid and fractionated with ethanol to recover the polypeptide-phospholipid-polysaccharide complex and the polysaccharide. These compounds precipitated from aqueous solution on the addition of 2 vol. ethanol and were stored till use in 75 % (v/v) ethanol in water at 0°.

The preparation of polysaccharides. The polysaccharides were prepared from the mixture of polypeptide-phospholipid-polysaccharide complex and polysaccharide (1 %, w/v, aqueous solution) by partial degradation with 1 % (v/v) acetic acid at 100°. After 4 hr. hydrolysis the acid-insoluble precipitate which formed was separated by centrifugation and the supernatant fluid cooled to 0° and extracted with ether. The material soluble in the aqueous phase proved to be polysaccharide. It was recovered from solution by the addition of 2 vol. ethanol and was repeatedly precipitated from aqueous solution by ethanol.

The degradation of the polysaccharide component of trichloroacetic acid extracts. Each polysaccharide solution was refluxed for 6 hr. in 0·25 N-HCl and then de-ionized in a mercury cathode de-salter until free from chloride and sulphate. This ion-free solution of hexoses was concentrated and analysed by chromatographic methods.

Chromatography. The descending method was employed. Whatman No. 4 filter paper was used in all experiments.

Solvents. The following three solvents were used in the analysis of each polysaccharide: *n*-butanol + acetic acid + water (4:1:5, v/v; Slotta & Primosigh, 1951); *n*-butanol + ethanol + water (10:1:2, v/v; Williams & Bevenue, 1951); ethyl acetate + acetic acid + water (3:1:3, v/v; Jermyn & Isherwood, 1949).

Identifying reagents. In recent years a number of rare or unidentified sugars have been isolated from bacterial polysaccharides (Lüderitz & Westphal, 1952; Dudman & Wilkinson, 1956; Davies, 1956; Strange & Dark, 1956). In order to be able to detect all classes of carbohydrate and to decrease the possibility of some sugars remaining unidentified, a wide range of identifying reagents was used. The identity of an unknown sugar was considered to be established when its R_G value corresponded with that of a known control in several different solvents and when its colour reactions with eight identifying reagents also corresponded with those of the same control. The following reagents were used in the chromatographic analysis of each polysaccharide: benzidine reagent (Horrocks, 1949); aniline hydrogen oxalate reagent (Horrocks & Manning, 1949); ammoniacal silver nitrate reagent (Partridge, 1948*a*); naphthoresorcinol reagent (Partridge, 1948*a*) modified as follows: 1 vol. 0·5 % (w/v) ethanolic naphthoresorcinol added to 1 vol. 0·1 N-HCl. By decreasing the amount of acid it was possible to prevent the charring of chromatograms which tends to occur when higher concentrations are used. The colour differentiation obtained with different sugar types was unimpaired); ninhydrin reagent (Partridge, 1948*a*); 3:5-dinitrosalicylic acid reagent (Williams & Bevenue, 1951); β-naphthylamine reagent (Novellie, 1950); iodine vapour reagent (Greenway, Kent & Whitehouse, 1953).

Special techniques. The separation of glucosamine and galactosamine by paper chromatography is not always easy. Two methods were used to differentiate these hexosamines. The first depends on the utilization of glucosamine as substrate by hexokinase in the presence of adenosine-5-triphosphate.

The method was described by Slein (1952). The second method, which is more efficient, depends on the conversion of 2-amino sugars to their corresponding parent pentoses by ninhydrin in pyridine buffer. The technique used was as described by Stoffyn & Jeanloz (1954) except that the sealed ampoules were heated at 100° for 1 hr. About 50 μg. of each hexosamine treated with ninhydrin reagent in pyridine buffer were applied to the chromatograms.

Two-dimensional chromatography and elution techniques were used to confirm the results obtained with the above methods.

RESULTS

The treatment of acetone-dried organisms of twelve Shigella strains with trichloroacetic acid resulted in each instance in the recovery of polypeptide-phospholipid-polysaccharide complex and polysaccharide. The amount of material so obtained varied from organism to organism, being as high as 6·7 % of the dry weight of bacteria with *Shigella flexneri* type 1a, and as low as 4·4 % of the dry weight with *S. flexneri* type 6.

The chemical nature of the trichloroacetic acid extracts

On partial hydrolytic degradation with acetic acid each trichloroacetic acid extract was shown to consist of three components. In their gross chemical and physical properties these components were analogous to those already studied by Morgan & Partridge (1940) and by Goebel *et al.* (1945). Component I was insoluble in acetic acid and ether and on prolonged hydrolysis with N-HCl yielded a number of amino acids but no monosaccharides; this component was considered to be a polypeptide. Component II was ether-soluble and contained phosphorus when examined by the method of Hanes & Isherwood (1949); it was considered to be a phospholipid. Component III was soluble in acetic acid and insoluble in ether. On hydrolysis with N-HCl it yielded a number of monosaccharides and no amino acids; it was considered to be a polysaccharide.

Chromatographic analysis

Each of the twelve antigenic complexes under investigation and their polypeptide, phospholipid and polysaccharide components was examined chromatographically, using the solvents and identifying reagents described above to demonstrate that they were not contaminated with free amino acids or sugars. The hydrolysed polypeptides were examined chromatographically and shown to be free from sugars, indicating that the polypeptides were free from polysaccharide contamination. Similarly, the hydrolysed polysaccharides were examined chromatographically and shown to be free from amino acids, indicating that the polysaccharides were free from polypeptide contamination. About 500 μg. of each component were applied to the chromatogram.

The hexose constituents of the Shigella polysaccharides

Shigella flexneri type 1a polysaccharide hydrolysate contained three monosaccharide constituents. Each of these constituents reacted with benzidine, aniline hydrogen oxalate, 3:5-dinitrosalicylic acid, ammoniacal silver nitrate and β-naphthylamine, giving the typical colour reactions of an aldose. The first constituent also gave a positive ninhydrin reaction and is therefore an amino sugar. It corresponded in R_G with a control of glucosamine hydrochloride in a number of different solvents and was utilized as substrate in the hexokinase reaction, thus confirming its identity. The second constituent did not react with the ninhydrin, naphthoresorcinol or iodine reagents but corresponded in R_G with a control of glucose in several different solvents; it also reacted as substrate in the hexokinase reaction, thus confirming its identity. The third constituent gave a positive naphthoresorcinol reaction and its colour reactions with benzidine and aniline hydrogen oxalate also suggested that it was a substituted pentose. It corresponded in R_G value with a control of rhamnose in several solvents. The results of a similar series of experiments with each of the other seven Flexner polysaccharide hydrolysates revealed that they likewise consisted of the same three compounds, namely glucosamine hydrochloride, glucose and rhamnose. The identity of the 2-amino sugar in the hydrolysate of *S. flexneri* type 2a polysaccharide was confirmed by demonstrating its utilization as substrate in the hexokinase reaction. The 2-amino sugars in the hydrolysates of the polysaccharides from *S. flexneri* types 3, 4a, 5 and 6 and from Flexner X and Flexner Y were converted to arabinose by the ninhydrin reaction in pyridine buffer. The 2-amino sugar present in these Flexner polysaccharides therefore seems most likely to be glucosamine hydrochloride.

By the same methods the hexoses present in the other Shigella polysaccharide hydrolysates were determined as follows: *Shigella shigae* polysaccharide hydrolysate contained glucosamine hydrochloride, galactose and rhamnose; *S. sonnei* polysaccharide hydrolysate contained glucosamine hydrochloride, galactose and glucose; *S. schmitzi* polysaccharide hydrolysate contained glucosamine hydrochloride, galactose, glucose and rhamnose; *S. alkalescens* polysaccharide hydrolysate contained glucosamine hydrochloride, galactosamine hydrochloride, glucose and galactose. The identity of the 2-amino sugars was confirmed by converting them to pentoses with ninhydrin reagent in pyridine buffer.

Table 1. *The constituent hexoses of the Shigella polysaccharides*

Shigella strain from which polysaccharide was extracted	Glucosamine	Galactose	Glucose	Rhamnose	Galactosamine
Shigella flexneri type 1a	+	−	+	+	−
S. flexneri type 2a	+	−	+	+	−
S. flexneri type 3	+	−	+	+	−
S. flexneri type 4a	+	−	+	+	−
S. flexneri type 5	+	−	+	+	−
S. flexneri type 6	+	−	+	+	−
Flexner X (Andrewes & Inman)	+	−	+	+	−
Flexner Y (Andrewes & Inman)	+	−	+	+	−
S. shigae	+	+	−	+	−
S. sonnei	+	+	+	−	−
S. schmitzi	+	+	+	+	−
S. alkalescens	+	+	+	−	+

Arabinose was formed from the 2-amino sugar present in each polysaccharide hydrolysate so that this hexosamine seems most likely to be glucosamine hydrochloride. In the case of the *Shigella alkalescens* polysaccharide hydrolysate, lyxose was also formed from the second 2-amino sugar so this hexosamine seems to be galactosamine. Control specimens of glucosamine and galactosamine hydrochlorides were treated with ninhydrin reagent in pyridine buffer as described above under identical experimental conditions to demonstrate their conversion to arabinose and lyxose respectively. Table 1 shows the constituent monosaccharides of the Shigella polysaccharides which have been studied in this investigation.

CONCLUSIONS

The eight Flexner polysaccharides investigated all possess identical monosaccharide constituents. The other Shigella polysaccharides differ in their monosaccharide constituents not only from the Flexner group as a whole but also from one another. In other words, the polysaccharides of the serologically heterogeneous Flexner group contain identical monosaccharides while the polysaccharides of the serologically homogeneous members of the genus *Shigella* contain different monosaccharides. The finding that *S. alkalescens* (NCTC 7925) polysaccharide contains galactosamine confirms the observation of Davies (1955). The absence of this hexosamine from the polysaccharides of the other Shigella types may be of taxonomic significance. It is of interest to note that the Shigella Commission Report (1951) removes this organism from the genus *Shigella* and places it in the genus *Escherichia* on the basis of serological evidence.

The chief value of the present work is that the number of Flexner polysaccharides examined has now been extended to include at least one from each Flexner type. In the case of the polysaccharides extracted from the other members of the genus *Shigella* the findings of previous workers have been confirmed using, in all but two instances, a different method of extraction. Goebel *et al.* (1945) reported the presence of a hexosamine (estimated as glucosamine) in the polysaccharides from *S. flexneri* types 1a, 2a, 3 and 6. Slein & Schnell (1952) and Slein (1952) identified glucosamine, glucose and rhamnose in the polysaccharide from a strain of *S. flexneri* type 3. These findings are confirmed here chromatographically by using a different method of polysaccharide extraction; the hexose constituents of the polysaccharides from strains of *S. flexneri* types 4a and 5 and Flexner X and Y are identified for the first time. The findings of Morgan (1931, 1932, 1936, 1937), Morgan & Partridge (1940, 1941) and Partridge (1948*b*) in respect of the hexose constituents of *S. shigae* polysaccharide are confirmed with a different strain of *S. shigae*. The polysaccharide from the strain of *S. sonnei* examined contains the same hexose constituents as the strain studied by Jesaitis & Goebel (1952) who used a different method of extraction. The polysaccharides from the strains of *S. alkalescens* and *S. schmitzi* examined contained the same hexose constituents as those examined by Davies (1955), using the same method of extraction.

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REFERENCES

Boivin, A. & Mesrobeanu, L. (1934). Remarques concernant la technique d'extraction du complexe polysaccharidique antigénique renfermé dans le bacille d'Aertrycke. *C.R. Soc. Biol., Paris*, **115**, 304.

Boivin, A. & Mesrobeanu, L. (1937). Recherches sur les toxines des bacilles dysentériques. Sur la nature et sur les propriétés biologiques des principes toxiques susceptibles de se recontrer dans les filtrats des cultures sur bouillon bacille de Shiga. *C.R. Soc. Biol., Paris*, **124**, 442.

Davies, D. A. L. (1955). The specific polysaccharides of some Gram-negative bacteria. *Biochem. J.* **59**, 696.

Davies, D. A. L. (1956). A specific polysaccharide of *Pasteurella pestis*. *Biochem. J.* **63**, 105.

Davies, D. A. L., Morgan, W. T. J. & Mosimann, W. (1954). Studies in immunochemistry. 13. Preparation and properties of the 'O' somatic antigen of *Shigella dysenteriae* (Shiga). *Biochem. J.* **56**, 572.

Davies, D. A. L., Morgan, W. T. J. & Record, R. B. (1955). Studies in immunochemistry. 15. The specific polysaccharide of the dominant 'O' somatic antigen of *Shigella dysenteriae*. *Biochem. J.* **60**, 290.

Dudman, W. F. & Wilkinson, J. F. (1956). The composition of the extracellular polysaccharide of *Aerobacter-Klebsiella* strains. *Biochem. J.* **62**, 289.

Girard, G. (1941). Absence d'antigène glucido-lipidique chez le bacille de la peste et le bacille de la pseudo-tuberculose des rongeurs. *C.R. Soc. Biol., Paris*, **135**, 1577.

Goebel, W. F., Binkley, F. & Perlman, E. (1945). Studies on the Flexner group of dysentery bacilli. *J. exp. Med.* **81**, 315.

Greenway, R. M., Kent, P. W. & Whitehouse, M. W. (1953). Detection of non-reducing carbohydrates in paper electrophoresis and chromatography. *Research, Lond.* **6**, Suppl. no. 1.

Hanes, C. S. & Isherwood, F. A. (1949). Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

Horrocks, R. H. (1949). Paper partition chromatography of reducing sugars with benzidine as a spraying reagent. *Nature, Lond.* **164**, 444.

Horrocks, R. H. & Manning, G. B. (1949). Partition chromatography on paper. Identification of reducing substances in urine. *Lancet*, i, 1042.

Jermyn, M. A. & Isherwood, F. A. (1949). Improved separation of sugars on the paper partition chromatogram. *Biochem. J.* **44**, 402.

Jesaitis, M. A. & Goebel, W. F. (1952). The chemical and antiviral properties of the somatic antigen of phase II *Shigella sonnei*. *J. exp. Med.* **96**, 409.

Lüderitz, O. & Westphal, O. (1952). Über bakterielle Reizstoffe: II Mitt.: Qualitative und quantitative papierchromatographische Bestimmung der Zuckerbausteine eines hochgereinigten Polysaccharid-pyrogens aus *Colibakterien*. *Z. Naturf.* **7b**, 548.

MacKenzie, G. M., Pike, R. M. & Swinney, R. E. (1940). Virulence of *Salmonella typhimurium*. *J. Bact.* **40**, 171.

MacPherson, C. R. (1956). Some observations on the antigenic structure of trichloroacetic acid extracts in Escherichia and the Paracolon group. *J. gen. Microbiol.* **14**, 520.

Morgan, W. T. J. (1931). A specific precipitating polysaccharide from *B. dysenteriae* (Shiga). *Brit. J. exp. Path.* **12**, 62.

Morgan, W. T. J. (1932). The estimation of small amounts of a specific bacterial polysaccharide by the induction of anaphylaxis. *Brit. J. exp. Path.* **13**, 342.

Morgan, W. T. J. (1936). Studies in immunochemistry. The preparation and properties of a specific polysaccharide from *B. dysenteriae* (Shiga). *Biochem. J.* **30**, 909.

Morgan, W. T. J. (1937). Studies in immunochemistry. The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). *Biochem. J.* **31**, 2003.

Morgan, W. T. J. & Partridge, S. M. (1940). Studies in immunochemistry. 4. The fractionation and nature of antigenic material isolated from *Bact. dysenteriae* (Shiga). *Biochem. J.* **34**, 169.

Morgan, W. T. J. & Partridge, S. M. (1941). Studies in immunochemistry. The use of phenol and of alkali in the degradation of antigenic material isolated from *Bact. dysenteriae* (Shiga). *Biochem. J.* **35**, 1140.

Novellie, L. (1950). An improved method of detecting sugars on paper chromatograms. *Nature, Lond.* **166**, 745.

Partridge, S. M. (1948*a*). Filter-paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.

Partridge, S. M. (1948*b*). Filter-paper partition chromatography of sugars. 2. An examination of the blood group A specific substance from hog gastric mucin and the specific polysaccharide of *Bacterium dysenteriae* (Shiga). *Biochem. J.* **42**, 251.

Shigella Commission Report (1951). See *International Bulletin of Bacteriological Nomenclature and Taxonomy*, 1951, **1**, 154.

Slein, M. W. (1952). A rapid method of distinguishing D-glucosamine from galactosamine in biological preparations. *Proc. Soc. exp. Biol., N.Y.* **80**, 646.

Slein, M. W. & Schnell, G. W. (1952). The specific polysaccharide of *Shigella flexneri*. *Bact. Proc.* p. 110.

Slein, M. W. & Schnell, G. W. (1953). An aldoheptose phosphate in a polysaccharide isolated from *Shigella flexneri*. *Proc. Soc. exp. Biol., N.Y.* **82**, 734.

Slotta, K. H. & Primosigh, J. (1951). Amino-acid composition of crotoxin. *Nature, Lond.* **168**, 696.

Stoffyn, P. J. & Jeanloz, R. W. (1954). Identification of amino-sugars by paper chromatography. *Arch. Biochem. Biophys.* **52**, 373.

Strange, R. E. & Dark, F. A. (1956). An unidentified amino-sugar present in cell walls and spores of various bacteria. *Nature, Lond.* **177**, 186.

Williams, K. T. & Bevenue, A. (1951). A simple technique for the identification of raffinose and sucrose by enzymatic hydrolysis on paper chromatograms and the subsequent separation of the hydrolysed products by paper chromatography. *Science*, **113**, 582.

(*Received* 22 *June* 1957)

McMillan, R. (1957). *J. gen. Microbiol.* 17, 658–677.

Morphogenesis and Polymorphism of *Ankistrodesmus* spp.

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SUMMARY: Pure cultures of *Ankistrodesmus* spp. showed continual morphological fluctuation when growing in complex organic media, and to a lesser (though detectable) extent in mineral media. Clonal populations were no less variable. In mineral media, variation of *A. braunii* was associated with the stationary phase of growth only, but in the complex organic test medium was seen in logarithmic phase as well. The normal processes governing cell morphogenesis are described for *A. braunii*. Their normal sequence is disrupted by increasing osmotic pressure of the medium in reverse of their normal order in time and of their hypothetical serial coupling to the metabolism concerned with cell growth. The final uncoupling, that of growth from initiation of cell division, is also accomplished by a wide range of C–N heterocyclic ring structures in very low concentration, which may indicate that nucleic acid metabolism is the system concerned at this point in the sequence.

During the course of a search for organic growth factors for algae of diverse taxonomic position, a strain of *Selenastrum* sp. was found to produce large numbers of morphologically variant cells when grown in the presence of small amounts of yeast extract in mineral medium. Variation was nearly as marked in a complete defined Lactobacillus medium containing nineteen amino acids and various other growth factors and metabolites (Table 1) even at relatively low concentrations.

Agardh (1820) introduced the term 'polymorphism' to describe the occurrence of morphological variants of a given form which simulate other related or unrelated species. Artari (1892) and Klebs (1896) included excellent critical reviews of the many observations of the phenomenon made during the nineteenth century. Various workers of the present century, beginning with Beijerinck (1904), attempted to apply the pure-culture methods of bacteriology in the study of algal polymorphism. The group of phycologists at the University of Geneva, headed by Chodat, was most active in this field. However, in spite of both Klebs's and Artari's recommendations for study of morphological changes in individual organisms in carefully controlled microcultures, the Geneva workers failed to clarify the essential question of inherent variability in clonal lines by investigating mass cultures at the time of maximal growth or later. Chodat (1913) at first interpreted his studies as proving glucose to be the direct cause of polymorphism, and on the basis of behaviour in glucose media attempted the classification of many 'elementary species' of the order Chlorococcales, some of which are still recognized by present-day taxonomists. Hofmann-Grobéty's (1912) results may now be interpreted as demonstrating that the high toxicity of glucose for *Raphidium minutum* Naeg. (*Selenastrum minutum* (Naeg.) Collins) permits survival of only rare individuals which are probably resistant mutants. Vischer (1920) considered the aberrant individuals in his cultures of *Ankistrodesmus braunii* to be due to inhibition of cytokinesis by hexoses. Both these investigators found peptone to be protective against toxicity of glucose, though peptone itself was highly toxic when used alone in low concentrations.

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The possibility of genetic causes of this commonly observed morphological variability of the Chlorococcales was not taken seriously at first by Chodat. He, like other nineteenth-century workers thought of 'mutations' as sudden phenotypic bursts of new, complete species. Oettli (1927) concluded that the morphological fluctuation he observed in five 'elementary species' of Ankistrodesmus was due to 'simple, non-hereditary modifications', but admitted he had not attempted to prove his notion, even when a population of elongate individuals appeared to arise from one of spherical individuals upon transfer to different media. Chodat later revised his thinking (1929) to propose stepwise 'generalized micromutation' of single characters as the explanation of repeatedly occurring pigment variants in *Chlorella rubescens*. However, up to his death in 1934, he had made no attempt to explain on this basis the nature of variation in cell-shape, nor to carry out the microculture studies urged by his critics.

The independent discovery of polymorphism in *Selenastrum* sp. growing in a defined organic medium suggested that an extensive re-investigation of the phenomenon in the light of modern biochemistry and genetics might disclose the extent of morphological variability to be expected in cultured strains of the simpler green algae, as well as some of the actual mechanisms of cellular morphogenesis. Further, low concentrations of common organic metabolites in solution should simulate natural ecological conditions, such as those in a pond in late summer, and elucidate the extent to which such factors might induce significant variations in wild populations of planktonic algae. Such naturally-occurring 'phenocopies' might well cause taxonomic confusion, and probably have done so in the past, whenever identification has been based on a few organisms found in a collection rather than on cultures isolated from the sample.

METHODS

Mass populations

Experiments were designed to test the effect of the defined organic medium (Table 1) and its fractional variants (Table 2) on stationary-phase cultures of mass populations; i.e. under conditions comparable to those of the Geneva studies. Only experiments with *Ankistrodesmus* spp. will be reported at the present time. Studies of *Selenastrum* spp. and *Scenedesmus* spp. were also begun, but were not extended beyond the first mass culture runs, as these organisms grew less rapidly and were more difficult to handle in centrifuging, dilution, etc., than *Ankistrodesmus* spp.

Table 1. *Composition of the complete defined organic medium used*

Constituent	Quantity/ 100 ml.	Constituent	Quantity/ 100 ml.
DL-Alanine	0·8 mg.	L-Tyrosine	0·2 mg.
L-Arginine	0·2 mg.	DL-Valine	1·0 mg.
L-Aspartic acid	0·6 mg.	Adenine sulphate	0·02 mg.
L-Cysteine	0·8 mg.	Guanine hydrochloride	0·02 mg.
L-Cystine	0·8 mg.	Uracil	0·02 mg.
L-Glutamic acid	1·0 mg.	Thiamine hydrochloride	0·4 μg.
L-Glycine	0·2 mg.	Riboflavin	0·4 μg.
DL-Histidine	0·4 mg.	Nicotinic acid	0·4 μg.
DL-Isoleucine	1·0 mg.	Biotin	0·008 μg.
DL-Leucine	1·0 mg.	Pyridoxine hydrochloride	0·8 μg.
L-Lysine	0·4 mg.	*p*-Aminobenzoic acid	0·08 μg.
DT-Methionine	0·8 mg.	Folic acid	0·016 μg.
DL-Phenylalanine	0·4 mg.	Calcium pantothenate	0·4 μg.
L-Proline	0·2 mg.	Glucose	0·04 g.
DL-Serine	0·4 mg.	Sodium acetate	0·024 g.
DL-Threonine	0·4 mg.	GFS* solution	To make 100 ml.
DL-Tryptophane	0·4 mg.		

* GFS, see Gerloff, Fitzgerald & Skoog, 1950.

Table 2. *Code for types of media*

Medium no.	Constituents
1±	Lacking or having only aa group 1 (monoamino monocarboxylic): gly., ala., leu., isol.
2±	Lacking or having only aa group 2 (monoamino dicarboxylic): asp., glut.
3±	Lacking or having only aa group 3 (diamino monocarboxylic): arg., lys.
4±	Lacking or having only aa group 4 (S-containing): cysti., cyste., meth.
5±	Lacking or having only aa group 5 (Ar): ϕala, tyr.
6±	Lacking or having only aa group 6 (heterocyclic): hist., pro., tryp.
7±	Lacking or having only vitamins: biotin, nicotinic acid, riboflavin, thiamine HCl, pyridoxine HCl, PABA, folic acid, Ca pantothenate
8±	Lacking N bases: adenine, guanine, uracil
9	Complete
10	Complete − glucose (i.e. + acetate alone)
11	Complete − acetate (i.e. + glucose alone)
12	Complete − acetate and glucose
13	GFS* alone
14	13 + yeast extract
15	13 + glucose
16	13 + acetate
17	13 + acetate and glucose
18	13 + all amino acids
19	Complete—all amino acids

* GFS, see Gerloff, Fitzgerald & Skoog, 1950.

Ankistrodesmus falcatus v. *acicularis* 101 and *A. falcatus* 188, both from the Indiana University Algal Culture Collection, Bloomington, Indiana, U.S.A., were the first test strains. In these and all subsequent experiments, culture vessels were glass-capped rather than plugged with cotton-wool, and all experimental cultures were run in duplicate. In the first run, suspensions of organisms from the morphologically most homogeneous stock cultures, as determined by quantitative count, were inoculated into the liquid test media and incubated for 3 weeks at 24° with continuous daylight fluorescent illumination of approximately 400 f.c. At the end of this time the suspended sediment from the experimental cultures was sampled and counted differentially. Long-term effects were studied by retaining all cultures from this run for a total of 150 days; while at 80 days cultures growing on media nos. 9, 12, 13 and 14 (of Table 2) were subcultured into the same media and the daughter cultures incubated for the remainder of the experimental period. Cultures which showed impressive changes from their earlier state or from their parent cultures on preliminary examination were differentially counted at the end of the 150-day period. Finally, cultures which had been selected for this count were inoculated into the test media and their daughter cultures counted at 21 days. 'GFS', or medium no. 13 of Table 2, which was the Gerloff, Fitzgerald & Skoog (1950) version of Chu's (1942) medium no. 10, and medium no. 14 (Difco yeast extract in a concentration of 0·1 g./100 ml. of GFS) were used as control media for all experiments.

Clonal isolates

Results of the experiments with mass populations pointed out the wisdom of Artari (1892) and Klebs (1896) in urging the study of variants as they arose from single organisms. The establishment of known clonal lines might rule out a process of diploidization, segregation and recombination if it were involved in the morphological variation.

Some technical difficulties arose in the course of attempts to use the more common isolation procedures, which revealed certain characteristics of these organisms. Ankistrodesmus showed very low viability when spread on agar plates. Later experiments with the micromanipulator confirmed the impression that spreading techniques were lethal because of the extreme fragility of the organisms during mechanical handling. The technique finally evolved for successful isolation of single organisms with the de Fonbrune micromanipulator is described elsewhere (McMillan, 1955). By this method clonal substrains were isolated from each of the strains used in the earlier experiments, as well as a strain of *Ankistrodesmus braunii*. The latter species was added to the study at this time in order to extend observations within the genus, and because of the detailed studies of polymorphism in this species by Oettli (1927), Vischer (1920) and Chodat & Malinesco (1893).

Each of the clones was inoculated in triplicate into GFS medium as soon as turbidity was grossly visible in the isolation tube, and the transfers examined at 22 days. The clone of *Ankistrodesmus braunii* was representative of the degree of variation found in all the cultures, and so was the only one counted quantitatively.

Microcultures

The continual morphological variance seen in the clonal cultures necessitated detailed analysis of the phenomenon from as many individual organisms as possible. Since micromanipulative isolation of large numbers of individuals was tedious and time-consuming, a method of microculturing of relatively large numbers of organisms was sought. The Levy haemacytometer chamber proved ideal for this purpose. Organisms from the desired source were prepared and diluted with culture medium to give a final concentration not greater than 5–6 × 10^4 organisms/ml. The suspension was introduced with a Pasteur pipette into the haemacytometer, which had previously been sterilized and fitted with a sterile no. 1 coverslip held in place by a film of sterile white vaseline along either edge. The chamber was completely filled, including the moats, and sealed with melted sterile vaseline except for a small space at one end of the transverse moat, which was left open to permit exchange of gases. The location of individuals was plotted on a map of the chamber at zero time, and their subsequent development followed at intervals as desired. Inoculated chambers were incubated in large moist chambers fitted with small beakers of sterile water containing Kleenex wicks, at the same illumination and temperature as used in earlier experiments. The tube used for inoculation of the microculture was routinely retained and incubated as a control. By this means, detailed observations were made of thousands of individuals in various media, and the development of their progeny followed for as long as two to three months. Photographs were made of organisms *in situ* or from the control tube cultures, on Kodachrome, Microfile, Adox KB-14 and Kodak Plus-X films, by using the Leica Micro-Ibso apparatus. AO × 45 achromatic and apochromatic and × 90 apochromatic objectives and × 10 and × 15 compensating oculars were used.

Normal development of Ankistrodesmus braunii *under photoautotrophic conditions*

An intensive study was made of normal development of organisms in GFS medium. Ages of inoculum varied from 42 hr. to 85 days. Organisms which were initially 'atypical' as well as 'typical' were followed throughout the entire growth cycle.

Effect of specific media upon development of log-phase cells

Carbohydrates. Since Chodat (1913) had indicted glucose as the cause of polymorphism, and our earlier experiments implicated the complete defined medium or some of its components, these media were tested in microcultures. Runs were set up in medium no. 9 (the complete medium), and glucose (medium no. 15) at 0·1–10·0 times its standard concentration in the complete medium (i.e. 0·04, 0·4, 2·0 and 4·0 %, w/v). The glucose media were made up in GFS (medium no. 13). Also, 4·0 % (w/v) glucose was made up in Difco yeast extract medium (medium no. 14) and in GFS medium containing 5 times the normal concentration of nitrate, as organic and inorganic N supplements, respectively, to test the further hypothesis of Chodat (1913, 1929) that an inadequate supply of N in the presence of glucose caused polymorphism. Controls for these experiments were both types of N supplement without glucose, as well as GFS medium. Fructose, mannose and galactose were tested at 4 % (w/v) to determine the degree of molecular specificity. Pentaerythritol in concentrations equimolar with the different glucose concentrations was used as a control for the effect of increased osmotic pressure, as suggested by Dr S. H. Hutner (personal communication). In addition to qualitative cytological observations in all these media, counts were made periodically in the glucose runs to determine the total population in each concentration in each chamber, and the total numbers of variants of all sorts.

Non-amino acid constituents of organic medium. These compounds were tested in tube culture only, as previous experiments had shown practical identity between tube and microcultures of the same age. All the substances listed in Table 1 with the exceptions of glucose and the amino acids were made up in GFS medium at 1·0, 2·5, 5·0 and 10·0 times their standard concentration in the complex medium. Samples from each tube were examined microscopically after 72 hr. growth under the usual conditions of illumination and temperature, for normally released progeny *versus* 'bulging' cells. All tubes were examined grossly for relative turbidity at 13 days, to ascertain whether adaptation had occurred in the course of several division cycles. Samples of approximately these ages, 72 hr. and 13 days, had proved to be definitive in earlier experiments, since 72 hr. is normally the middle of the log phase and 13 days is in the normal stationary phase.

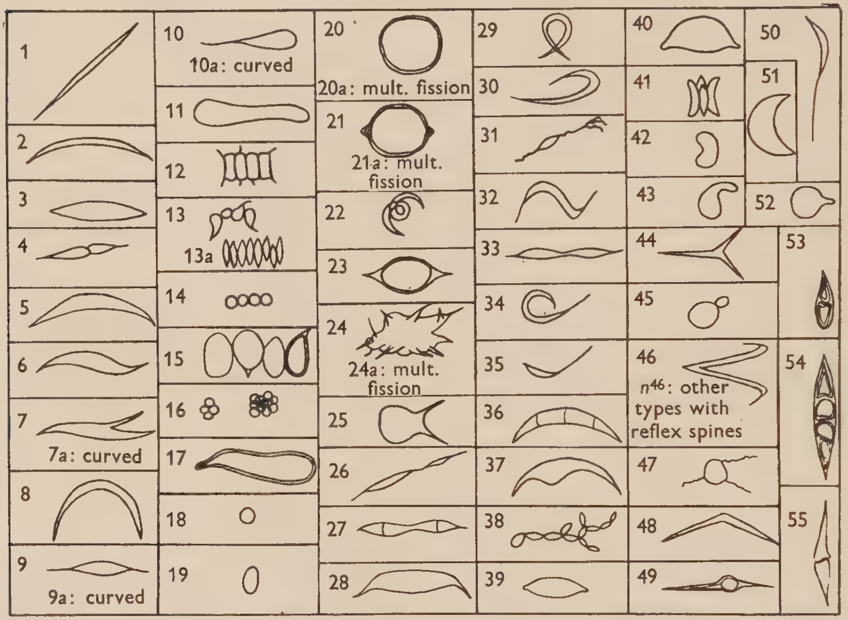
Purines, pyrimidines and related compounds. Tube cultures were set up at different concentrations of the following compounds: imidazole, pyridine, bipyridine, pyridoxine hydrochloride, adenine, guanine, xanthine, cytosine, uracil, thymine and thiamine hydrochloride. All compounds were tested in duplicate tube cultures at concentrations of 10, 5, 2·5 and 1 μg./ml., unless otherwise noted in Table 5. Control cultures were run in GFS medium. Samples from both experimental and control tubes were examined at 96 hr. and 280 hr. after incubation under the usual conditions.

RESULTS

Mass populations

Cell-types which were phenocopies of many recognized taxa of the Choloro-coccales and other forms were found in all daughter cultures of the first group of experiments at one time or another and in the various media, in spite of the selection of inocula for maximum homogeneity and predominance of species-types. Many of the variants bore no resemblance to the parental types (Fig. 1). The distribution of these cell-types in representative experiments is shown in Figs. 2 and 3. It was evident that the morphological variability of *Ankistrodesmus* spp. (as well as of the other forms not included in this report) was as great as reported by the early workers, and sufficiently remarkable to warrant further study. The correlation of particular variant types with any given culture medium was not clear-cut. Rather than extensive attempts at such a correlation, either by statistical means or by repeated experiments of this type, an investigation of the basic biological causes of the variation was chosen.

In long-term studies of the same substrains, the proportion of variants to 'parental' species-types did not appear to be constant (Table 3). Attempts to induce variant populations to revert to species-type by return to mineral medium were not uniformly successful; in fact, on three separate occasions (twice within one substrain) a variant became the predominant form and remained constantly so for some time. At present, it may be postulated that two independent mutations occurred in these strains, closely associated in time: one for modified cell shape (through alteration of metabolic pathways involved in cell elongation, cytokinesis, cell-wall formation, etc.) and one for metabolic efficiency. Unfortunately it has not been possible to test this hypothesis, for these strains were lost because of contamination, as were most of the substrains of the first experiments. However, this evidence of spontaneous alteration in populations is of considerable taxonomic interest. The clones of all three strains of *Ankistrodesmus*, when isolated, appeared fully as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n46: other types with reflex spines
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Fig. 1. Cumulative record of all morphological types seen in all cultures of all strains* in the course of studies of mass populations. Since the average size of individuals varied considerably within each strain as well as between strains, these outline sketches are of various magnifications.

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* Strain no. 1 = *Ankistrodesmus falcatus* v. *acicularis* 101 (Indiana University Algal Culture Collection).
2 = *Ankistrodesmus falcatus* 188 (Indiana University Algal Culture Collection).
3 = *Selenastrum* sp. 235 (Indiana University Algal Culture Collection).
4 = *Selenastrum* sp. 9 (McMillan isolate).
5 = *Scenedesmus dimorphus* 5 (McMillan isolate).
6 = *Scenedesmus quadricauda* 83 (Indiana University Algal Culture Collection).
7 = *Ankistrodesmus braunii* 245 (Indiana University Algal Culture Collection) (includes McMillan clonal isolate 245-St 1.1).

	Key to occurrence of morphological types		
Type no.	Species-type or originally predominant in strain no.	Predominance shifted to this type in strain no.	Seen occasionally in strains no.
1	1	2	All except 5
2	2	1	All except 5 and 6
3	7	—	All
4	—	—	All
5	—	1	All
6	—	—	All except 5 and 6
7	—	1	All
8	3 and 4	2	All except 5 and 6
9	—	1 and 2	All except 5
10	—	—	All
11	—	—	All
12	6 (species-type)	—	All except 1 and 2
13	5	—	All except 1 and 4
14	—	—	All except 2 and 3
15	—	—	3, 5 and 6
16	—	—	3, 5 and 6
17	—	—	All except 2 and 6
18	6 (original count)	—	All
19	6 (original count)	5	All
20	—	—	All
21	—	—	All
22	—	—	1, 2, 3 and 4
23	—	—	All
24	—	—	All except 5 and 6
25	—	—	2, 3, 4 and 5
26	—	1	1, 2, 3 and 7
27	—	—	All except 1
28	—	—	All except 1 and 2
29	—	—	2, 3 and 4
30	—	—	2, 3 and 4
31	—	—	4
32	—	—	3, 4
33	—	—	All except 5 and 6
34	—	—	2, 3 and 4
35	—	—	2, 3 and 4
36	—	—	2, 3, 4 and 7
38	—	—	4, 6
39	—	5 and 6	3, 7
40	—	—	5, 6
41	—	—	3, 5 and 6
42	—	—	All except 1
43	—	—	All except 1
44	—	—	All except 5 and 6
45	—	—	4
46	—	—	1, 2, 3 and 4
47	—	1	All except 5 and 6
48	—	—	1
49	—	—	1, 2
50	—	—	2, 3 and 4
51	—	—	2, 3 and 4
52	—	—	All except 1
53	—	—	7
54	—	—	7
55	—	—	All except 5 and 6

variable as the mass populations (Table 4). Most of the variant types were present in detectable numbers in the first transfer of each clone, though this variation was less marked in GFS medium than in organic media.

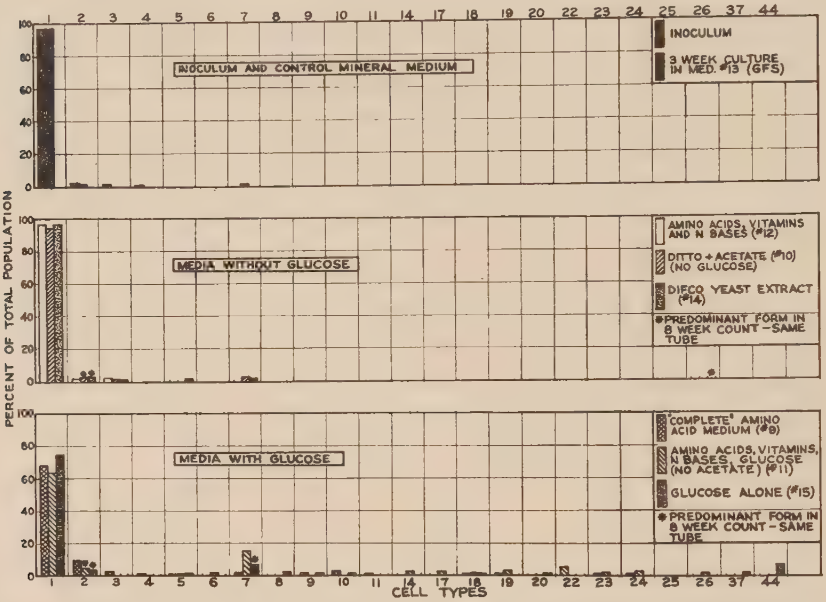


Fig. 2. Distribution of cell-types of Fig. 1, in representative runs of mass population studies of *Ankistrodesmus falcatus* v. *acicularis* 101 (Indiana University Algal Culture Collection). Inocula were prepared by pooling and centrifugally washing 3-week cultures. Cultures were differentially counted after 3 weeks of growth.

Phases of growth-cycle and variants

Two facts became evident during the course of the growth studies of *Ankistrodesmus braunii*. First, at the size inoculum chosen for the long-term studies, organisms were detectable at zero time and for a few hours thereafter; but there followed, in all cultures, a considerable lag period during which no growth was detectable, even under the low power of the dissecting microscope. Finally, about the sixth to tenth day, organisms were detectable and quantitative counts, discontinued after the first day, could be resumed (Fig. 4). The degree of decrease in direct count and length of lag phase varied inversely with the size of the inoculum (Fig. 5), except where organisms were concentrated by centrifugation and an inoculum larger than the normal stationary-phase density was used. The logarithmic phase of growth was characterized by several spurts, each followed by a macroscopic decrease in turbidity and marked decrease in the direct count by the microscope, together with evidence of lysis of organisms.

Secondly, in all the media chosen for this experiment as representative of the classes studied previously, morphology of the organism was remarkably homogeneous and of the species-type throughout the detectable logarithmic phase, with one exception, namely, in the complete medium (containing glucose and amino acids; medium no. 9) large numbers of bizarre forms were present even in young actively growing cultures.

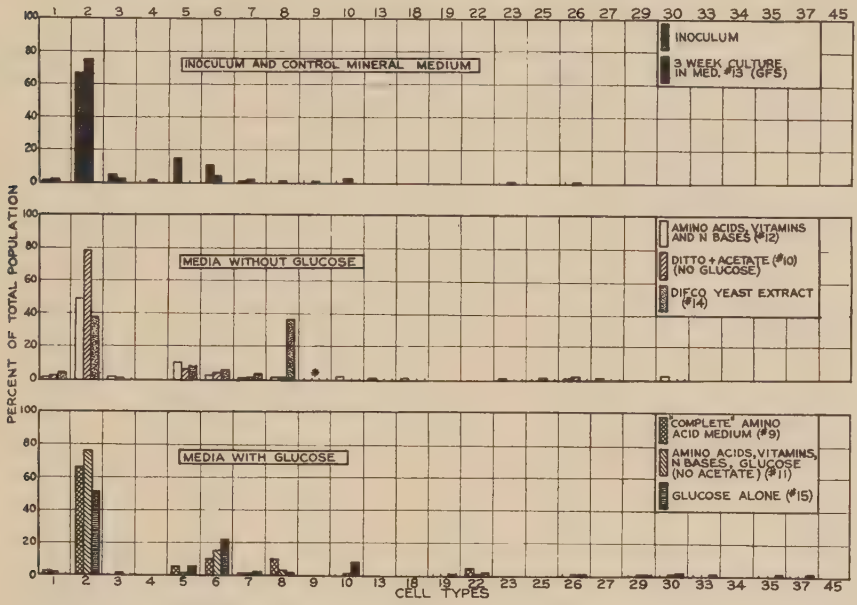


Fig. 3. **Distribution of cell-types of Fig. 1, in representative runs of mass population studies of *Ankistrodesmus falcatus* 188 (Indiana University Algal Culture Collection). Inocula were prepared by pooling and centrifugally washing 3-week cultures. Cultures were differentially counted after 3 weeks of growth.**

In the other media, variant forms first appeared in the negative acceleration phase, and gradually increased in number throughout the later life of the culture. This was interpreted as indicating that substances which accumulated during growth caused the increasing variation in ageing cultures. An alternative hypothesis was that heretofore unreported sexual processes were causing genetic recombination and redistribution of genes in the population.

The characteristics of growth in this experiment were remarkably similar in the two media with N supplement (media no. 9 and 14); and different from those without (media no. 13 and 15). The curves illustrating this (Fig. 4) are separated by two log cycles for better visibility. In the unsupplemented media, growth stopped abruptly at approximately 10^7 organisms/ml. after about three to five major bursts of growth, each followed by lysis and quiescence of organisms before the next series of cleavages. In the N-supplemented media, however, growth never terminated;—rather, the number of organisms increased slowly during the entire course of the 'stationary' phase, giving final counts two to five times as high as in the non-supplemented media. In all media the degree of initial decrease in count, duration of log phase and rate of increase in logarithmic phase were approximately the same.

Table 3. *Stable and shifting predominance in populations of* Ankistrodesmus falcatus *on long-term incubation and successive subculture. Cell-types as percentage of total population*

		Strain and conditions of growth				
Culture conditions	Cell-type no.	101-A 70 days—medium 14	101-E 150 days—medium 14	101-G 70 days—medium 9	188-B 150 days—medium 14	188-F 70 days—medium 12
Original inoculum	1	96·8	96·8	96·8	0·69	0·69
	2	1·8	1·8	1·8	66·9	66·9
	9	—	—	0·0	—	—
21 days on GFS med. (control)	1	97·1	97·1	97·1	0·56	0·56
	2	1·8	1·8	1·8	75·84	75·84
	9	—	—	0·0	—	—
1—21 days on this med. (control)	1	96·9	96·9	68·7	4·76	1·67
	2	0·88	0·88	9·88	38·0	49·04
	9	—	—	1·77	—	—
2—150 days on this med.–duplicate of *1*	1	—	70·75	—	0·0	—
	2	—	10·38	—	36·0	—
	9	—	—	—	—	—
3—70-day-old subculture from *1*	1	0·52	—	0·0	—	87·14
	2	15·18*	—	80·0	—	10·0
	9	—	—	0·0	—	—
2 or *3* washed, resusp. in fresh min. med., inc. 21 days	1	1·0	75·31	0·0	0·0	91·0
	2	46·0	3·78	64·0	44·0	0·0
	9	—	—	1·0	—	—
21-day subcult. from *2* or *3* (this med.)	1	5·21	91·0	0·0	1·0	95·0
	2	44·79	0·5	78·0	58·0	1·0
	9	—	—	0·0	—	—
21-day trans. of *2* or *3* to min. med.	1	1·5	92·0	1·06	0·0	96·0
	2	62·0	4·0	88·23	70·0	1·0
	9	—	—	4·26	—	—
Comments		Type 1 up to 88·5 % in some transfers	—	Type 9 = 69·5 % on transfer of *3* to yeast; remained constant	—	—

* Predominantly types 5 and 47.

Table 4. *Variation in individual morphology within clone of* Ankistrodesmus braunii, 22 *days' growth in mineral medium, first transfer after isolation*

Cell-type	Percentage of total population	Cell-type	Percentage of total population
2^{46}	0·2	28	0·2
3	90·8	39	1·0
7	1·0	50	0·4
10	0·4	53	4·4
18	0·2	54	0·8
19	0·2	55	0·2
24	0·2		

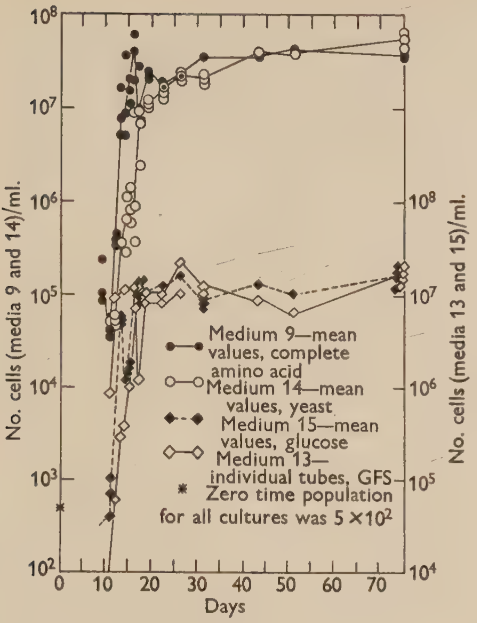


Fig. 4

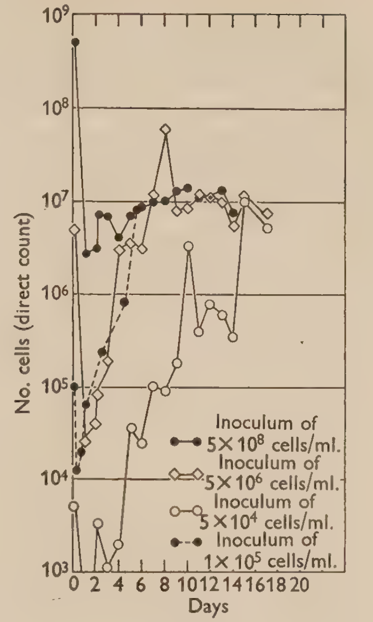


Fig. 5

Fig. 4. Growth curves in tube cultures of *Ankistrodesmus braunii* 245-St 1.1, in various media at 400 f.c. continuous illumination and 24°. Curves are drawn for means of samples, plus and minus standard deviation. Counts during the first 10 days of growth were not obtainable because of mass lysis (see text). Inoculum was prepared by pooling and centrifugally washing 3-week cultures.

Fig. 5. Growth curves in tube cultures of *Ankistrodesmus braunii* 245-St 1.1, at 400 f.c. continuous illumination and 24°, from inocula of varying size. The inoculum of 5×10^8 organisms/ml. was obtained by pooling and concentrating several cultures. All inocula were prepared by pooling and centrifugally washing 3-week cultures.

Normal development of Ankistrodesmus braunii

Anatomy of organisms. Ankistrodesmus braunii in the logarithmic phase of growth in mineral medium exhibits some features not clearly described previously. Typical individuals are shown in Pl. 1. They are fusiform, with a laminate, parietal chloroplast which extends nearly to each apex and about two-thirds around the periphery, much as in Ulothrix, etc. The chloroplast is capable of shifting position within the cell, so that it will come to lie in the bottom even when the individual is rolled over. At the equator of cell and chloroplast a prominent pyrenoid is located, usually surrounded by bead-like granules. Chains of granules ordinarily extend from the edge of the pyrenoid diagonally across the cell to the opposite wall, in the direction of either apex. Many conspicuous granules, crystals within vacuoles, etc., are usually present. The protoplast usually does not extend to the apices.

Division and growth (Pls. 1–3). In the early log-phase, the apex of the cell wall appears to soften and/or to excrete a slime, as the first indication of growth after introduction into fresh medium. This material is often of considerable volume and of a brownish colour, and may act as a holdfast when a suitable substratum is present; but its secretion does not depend upon availability of attachment. Occasionally only one apex will develop a disk; and in spineless individuals (as in those arising from exsporulation) the apical material is often nearly or completely aborted. Elaboration of the apical material and growth of the organism are followed by the first cleavage, which begins at the pyrenoid and shortly after at a point directly opposite. The first cleavage furrow divides both protoplast and pyrenoid equally. It has not yet been possible to demonstrate a nucleus conclusively, in this or subsequent divisions.

The second cleavage furrows occur simultaneously, midway between the first furrow and either apex, within 1–2 hr. of the first furrow and parallel to it. The fate of the pyrenoid is difficult to trace in this and subsequent cleavages. Following second cleavage, the daughter cells ordinarily divide again with the next 2–3 hr. while continuing to grow; the average organism contains eight closely packed daughters, each having the shape impressed upon it by its neighbours and the confining mother cell-wall. The last set of furrows tends to be oblique to the transverse axis rather than parallel to it, so that many daughters are quite triangular at this time. Each of the progeny begins to elongate and grow in a common, apparently predetermined direction parallel to the long axis, resulting in a spiral configuration. Elongation corrects the cramped shape of the cells and causes the old cell-wall to split off as two empty, conical caps which may be retained by the growing cluster for some time. The newly liberated daughters grow for a period of 10–20 hr. before the first cleavage of the next division cycle. Division in progeny of a given mother tends to remain approximately synchronous for two or three division cycles. However, in any culture chamber some individuals produce a few daughters which may initiate the next cycle or continue the old one while elongating, spiralling and bursting the original wall.

Ageing and sporulation (Pl. 4). After four to six division-cycles have been completed, the daughter clusters or microcolonies tend to fall apart into discrete individuals. The secretion of apical material has steadily decreased with successive division cycles. At about the sixth cycle after inoculation, the chloroplasts become granular and oil droplets begin to appear in the cytoplasm. Both these phenomena obscure the pyrenoid of mature individuals. Organisms of this age were the only ones studied by many of the early workers; hence their description of this form as lacking a pyrenoid (Artari, 1892; Chodat, 1909, 1913). At this time many individuals do not elongate after division, but stay packed in the original cleavage configurations. Such individuals soon secrete heavy secondary walls and become aplanospores, as described by Klebs (1883) and Artari (1892). Still other vegetative cells form aplanospores without dividing, by rounding up the protoplast within the cell wall and forming a secondary internal wall. Such organisms have large empty spaces in the apices. As a sporulating culture ages, both spores and the remaining vegetative cells gradually lose the chlorophylls and all visible chloroplast structures and become filled with large yellow to orange fatty droplets. Many of the spores appear to retain the ability to divide vegetatively, liberating small spherical cells, thus explaining the report of Beijerinck (1890) concerning the existence of a 'Chlorella cycle'. This cycle was particularly notable in the N-supplemented media, where the loss of green pigments was greatly retarded as well. Apparently in such cultures the mechanism of division is uncoupled from that of elongation.

Exsporulation (Pl. 5). Artari (1892) first observed the development of normal organisms from spores when he transferred dried spore clusters to fresh media in hanging-drop preparations. The pale, oil-packed organisms first undergo a rejuvenescence of pigments and chloroplasts essentially in the reverse order of events in the late stationary phase which led to bleaching and excessive fat deposition. Restoration of the smooth dark-green chloroplast may take from 2 to 5 days after inoculation into the culture chamber, and does not necessarily proceed at the same rate in all individuals of a spore cluster. Growth of the protoplast, after chloroplast function is restored, ruptures the spore case, in many instances before cleavage. The individuals liberated are approximately spherical when they originate from intercalary cells and conical to pisciform when originating from the apices. Frequently cleavage occurs while the germinating spore remains within the spore case. At any rate, elongation following cleavage produces tightly packed, coiled organisms, which soon burst the cell wall and retain their cramped form for only a short time; the normal events of growth and further elongation permit these cells to attain fairly normal shape, though many retain rather blunt apices. Progeny of the second division cycle are fully normal.

Abnormalities of spore germination and development are occasionally encountered, especially in some of the organic media. A few spores grow very large without cleavage and undergo a median constriction, which has never been seen to go to completion and give rise to two individuals. Other spores, after cleavage, liberate individuals which remain spherical, immediately lay down heavy spore walls, and repeat the process. The result is a cluster of cells in a *Protococcus*-like formation; only the original apical cells retain spines.

Abnormal vegetative cells. Most of the deviants from normal vegetative cells or stages associated with spores, fall into the category of triangular or multispinous cells. A small number of these is always present, especially from the third or fourth division cycle and later. Some of these may result from partial inhibition of cytokinesis, but many are apparently the product of the cramped configuration of daughters after cleavage. The cells located in the apices of the mother are most likely to be triangular or three-pronged in shape. Some of the progeny may retain this morphology, not because of genetic causes (though individual organisms might, of course, undergo a mutation) but usually because some of the plastic daughters are in turn pressed into the mould of the mother cell wall. The majority of progeny of a triangular individual is always of the species-type.

Lysis. Unpredictable and as yet uncontrollable numbers of organisms undergo spontaneous lysis even in control cultures (Pl. 6). This was the phenomenon which caused periodic decreases or plateaux in the net rate of increase in direct total count in the growth studies. At times cultures may be maintained for long periods in frequent serial transfer with little or no evidence of lysis; at other times, this phenomenon appears like an epidemic. The majority of such organisms are seen in microculture to undergo apparently normal cleavage corresponding to the first division-cycle after inoculation, but without elongation and spiralling of daughters. Usually there are two or more additional cleavages, with a final yield of 64 or 128 daughters. In 48–72 hr. after inoculation, a weak point develops at the equator in the cell wall of the mother, and the daughters are liberated in a shower of protoplasmic clumps. Some individuals do not liberate formed clumps, but retain most of the cleavage products except for a slight ooze. These stages are reminiscent of abortive attempts at zoospore production in other algae.

Development in the presence of specific compounds

Effect of carbohydrates (Pls. 7 and 8). Only the standard concentration of glucose in the complex medium (0·04 %, w/v) permitted normal growth as compared with the controls, when the total numbers of organisms were counted in each chamber (Fig. 6). However, contrary to the earlier experiments, even this concentration gave significant numbers of variant shapes, of the types shown in Pl. 7, until the fourth day after inoculation. In the early runs, very young cultures in this medium were not examined because of great losses of organisms by lysis in all cultures. In microcultures the whole population appeared to adapt in the course of the first few division cycles, finally liberating only normally elongated daughters; the slight lag in growth as compared to the controls was corrected at this time.

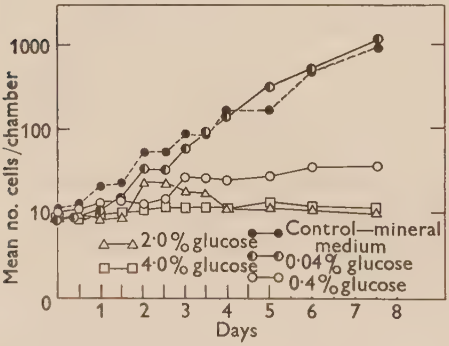


Fig. 6

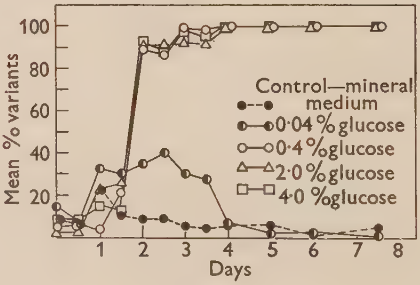
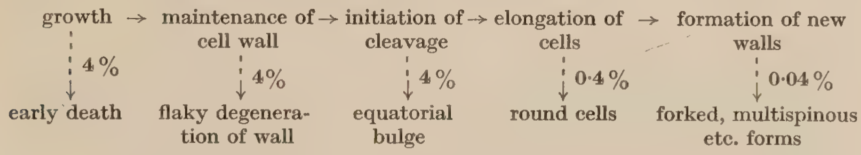


Fig. 7

Fig. 6. The effect of varying concentrations of glucose upon growth of *Ankistrodesmus braunii* 245-St 1.1 in haemacytometer cultures. Counts were made of the total number of organisms in each chamber at intervals after inoculation. Inocula were prepared by diluting 1/50 the pooled sediment of three 72 hr. cultures.

Fig. 7. The effect of varying concentrations of glucose upon morphology of *Ankistrodesmus braunii* 245-St 1.1 in haemacytometer cultures. Counts were made of the total number of normal organisms and deviants of any sort in each chamber at intervals after inoculation. Inocula were prepared by diluting 1/50 the pooled sediment of three 72 hr. cultures.

In the higher concentrations of glucose, the populations were more aberrant as the concentration increased (Fig. 7). Division was retarded at 0·4 % (w/v) and produced daughters which appeared incapable of elongation. At 2·0% (w/v) this effect was more pronounced, the resulting population consisting mostly of spherical, granular forms. A slight initial increase in numbers was followed by lysis of most organisms in what would have been the next division cycle. At 4·0% (w/v) glucose appeared to inhibit altogether the ability to release daughters after cleavage. Many individuals lysed at an early stage, but many of those which survived went on to grow without further cleavage, producing a great bulge at the equator which continued to increase for as long as five or six weeks. All these stages were shown in Vischer's (1920) photographs. The quantitative series of effects of glucose may represent a serial uncoupling of the sequences related to cell division and release of normal daughters, similar to the sequences proposed for some of the yeast-like fungi by Nickerson (1948, 1953, 1954). They may be summarized diagrammatically as follows, where solid arrows show normal processes, and broken arrows show inhibition and results. Percentages shown are concentrations of glucose (w/v):



These effects were shown to be non-specific by growing organisms in the other hexoses and in penta-erythritol. Penta-erythritol in a range of concentrations equimolar with those of glucose used earlier gave precisely comparable effects, except that the time required was longer; for example, the 'great bulge' stage required about 4 weeks for initiation, as compared to 3–4 days in glucose. This difference may have been due to a difference in permeability to the two compounds. It was concluded from these experiments that the effect was an osmotic one, and comparable to osmotic effects upon protistan cell division noted by other workers (Whitfield & Murray, 1956; Schröder, 1954). In all cases, the possibility of selection of morphological mutants was ruled out repeatedly by transfer of organisms from experimental media to GFS medium; in microculture, all viable individuals produced daughters capable of elongation and other normal morphogenetic activities in the first or second division cycles.

Effect of various other compounds. Some non-amino acid components of the original organic test medium at different concentrations gave essentially the same effects in extremely low (*c.* 10^{-7}—10^{-8} M) concentration as did the highest concentration of glucose ($2{\cdot}2 \times 10^{-2}$ M) (Table 5). Here, the effect could hardly have been osmotic, but might have been the result of blockage of specific metabolic pathways involved in morphogenesis. The effect appeared to be relatively non-specific, since the effective compounds were in the general category of C- and N-heterocyclic rings. The effective range of all the active compounds of this study is comparable to that of thymine antagonists for a thymineless strain of *Escherichia coli* (Cohen & Barner, 1956). This suggests that the nucleic acid-cobalamine-folic acid metabolic network may be one of

Table 5. *Effective levels of inhibition by heterocyclic compounds*

Substance	More than (μg./ml.)	Less than (μg./ml.)	Cytology
Control	—	—	Normal early stationary: fat deposition, sporulation
Azoles			
Imidazole	20	40	Normal cells but sparse
Pyridines			
Pyridine	10	20	Normal cells but sparse
Bipyridine	5	10	5 μg.: club-shaped, bulgy and normal cells; 10 μg.: no bulge—complete arrest of inoculum cells
Pyridoxine	40	?	Normal cells, slightly depressed growth
Purines			
Adenine	5	10	Small, swollen, bleached (arrested young 'monsters')
Guanine	?	1	Small, swollen, bleached (arrested young 'monsters')
Xanthine	1	2·5	Large, round 'monsters'
Pyrimidines			
Uracil	5	10	Bleached, lysing, arrested young 'monsters'
Thiamine HCl	10	20	Larger, round, lysed 'monsters'
Thymine	10*	?	Normal, early log-phase cells*

* KOH used to neutralize solution; may have resulted in hypertonicity at this concentration.

the systems involved. These and related compounds should be further screened to distinguish between quantitative parallel sensitivity of these sequences to the osmotic sequences, and the possibility that such compounds have an effect only upon metabolic pathways involved in some specific processes, such as cell elongation.

These studies were begun in the laboratories of the Department of Nutrition and Metabolism, Northwestern University Medical School, and completed in the Department of Biological Sciences, Northwestern University, in part with the aid of a grant to Dr L. H. Tiffany from the National Science Foundation of the United States. A portion of the material reported here was included in a dissertation submitted to the Graduate School of Northwestern University in partial fulfilment of requirements for the degree of Doctor of Philosophy. The author is gratefully indebted to Dr G. H. Mickey and Dr L. H. Tiffany for directing this research, and for their many helpful criticisms and suggestions; and to Miss Mary A. Strain for reading the manuscript.

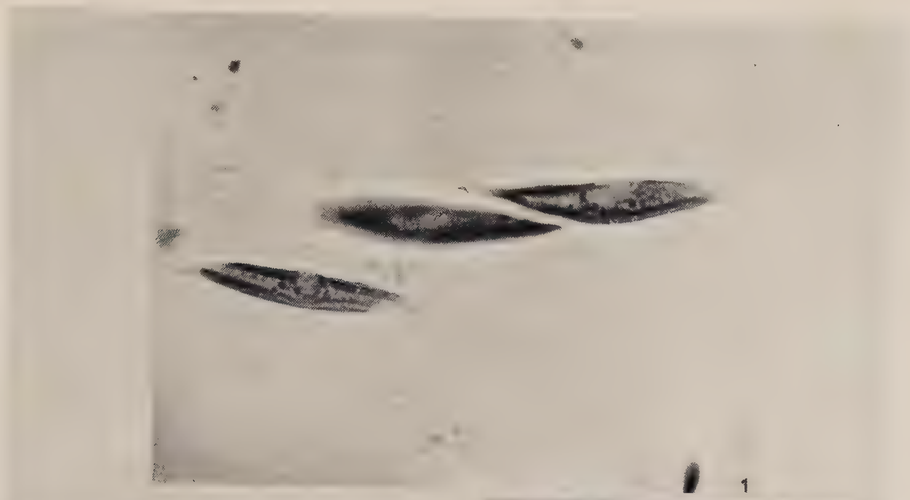
REFERENCES

AGARDH, C. A. (1820). De metamorphosi Algarum. In *Isis, oder Encyclopaedische Zeitung*, col. 644–654.

ARTARI, A. (1892). Untersuchungen über Entwicklung und Systematik einiger Protococcoideen. *Bull. Soc. imp. Nat. Moscou*, **6**, 222.

BEIJERINCK, M. W. (1890). Kulturversuche mit Zoochlorellen, Lichengonidien und anderen niederen Algen. *Bot. Ztg.* **48**, 725.

BEIJERINCK, M. W. (1904). *Chlorella variegata*, ein bunter Mikrobe. *Rec. Trav. bot. néerl.* **1**, 14.



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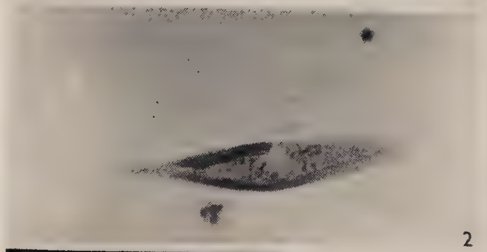

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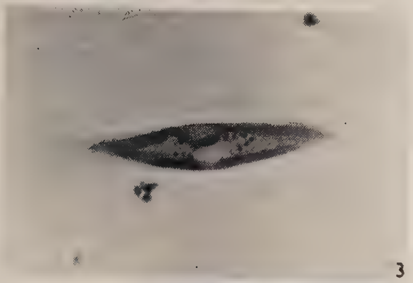

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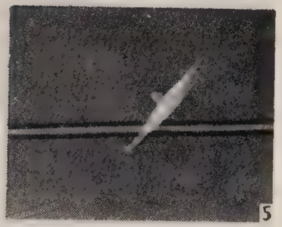

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R. McMillan—Morphogenesis of ankistrodesmus. Plate 1

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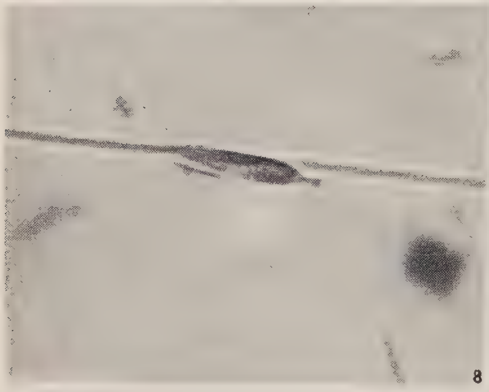

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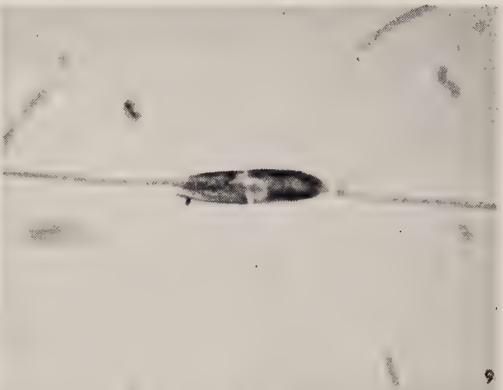

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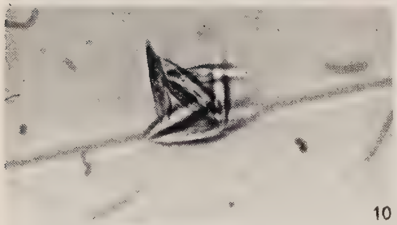

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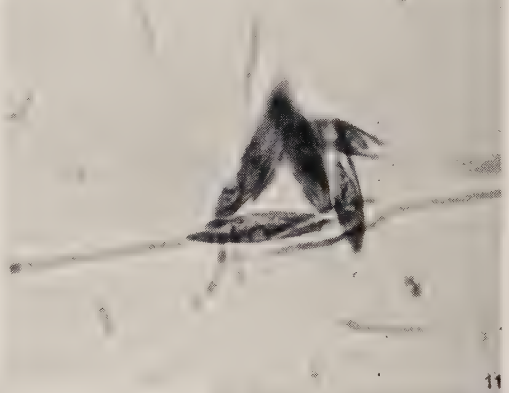

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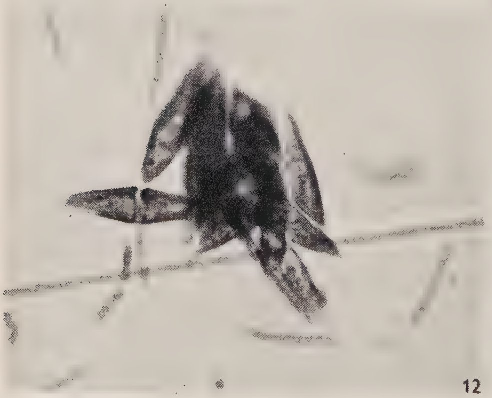

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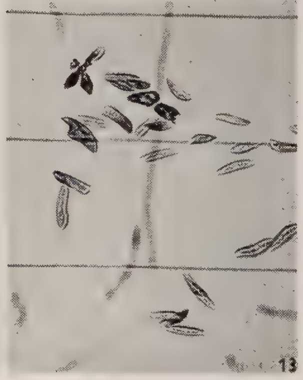

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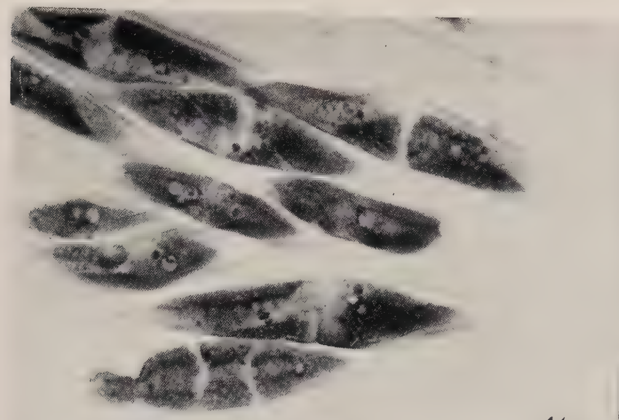
R. McMillan—Morphogenesis of ankistrodesmus. Plate 2



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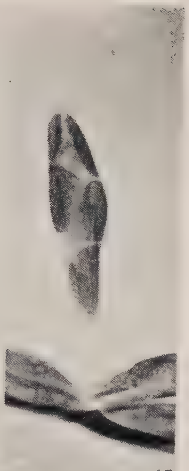

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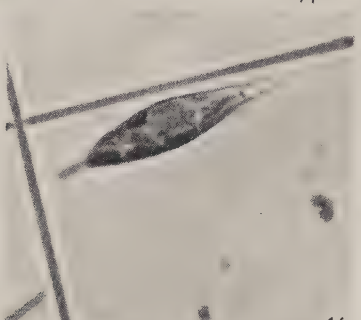

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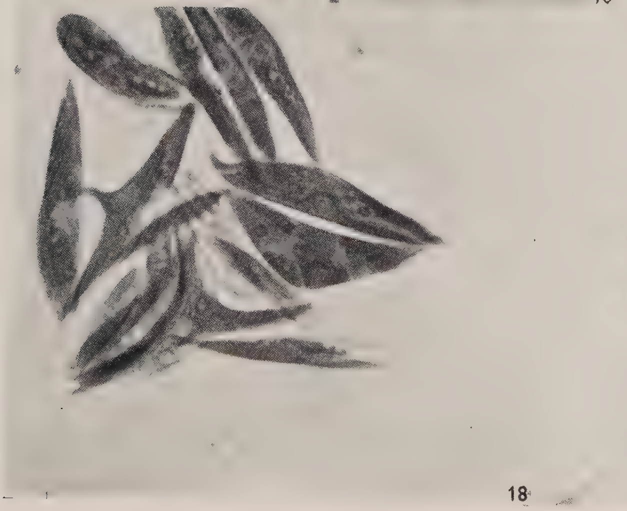

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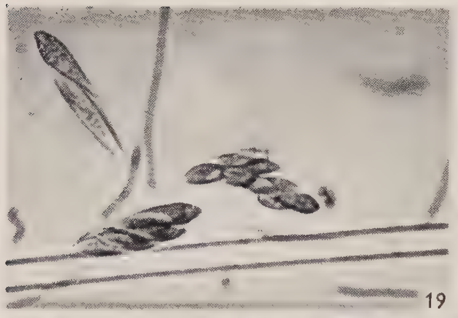
R. McMillan—Morphogenesis of ankistrodesmus. Plate 3



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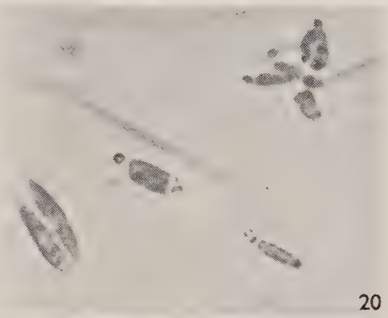

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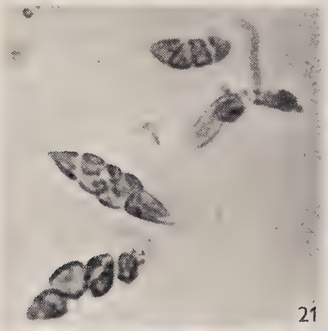

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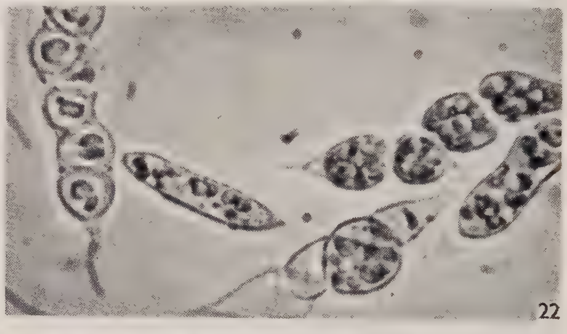

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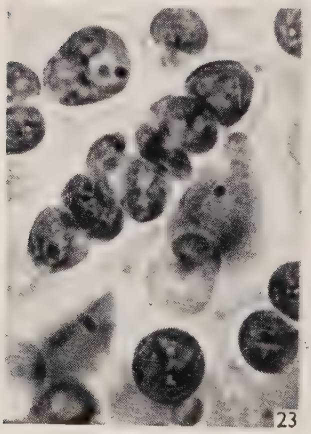

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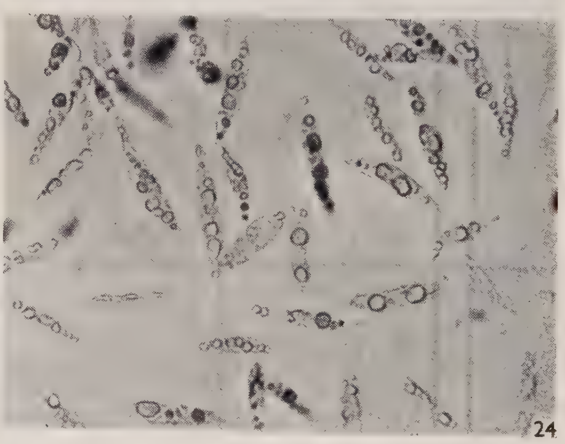

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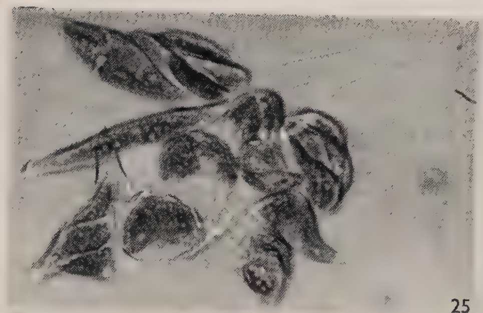

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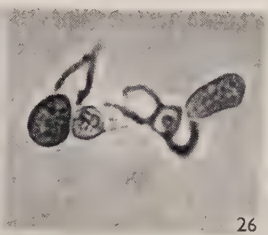

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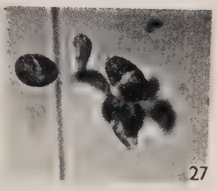

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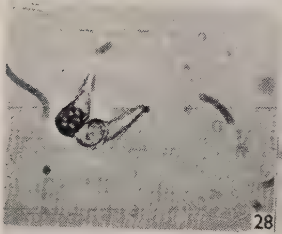

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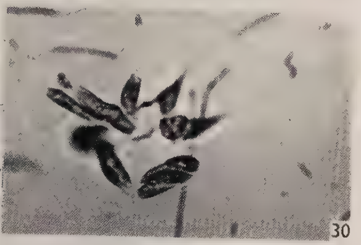

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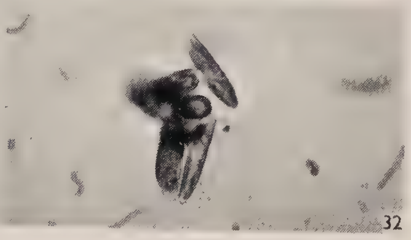

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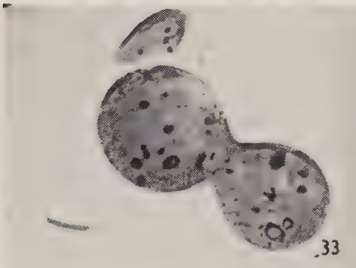
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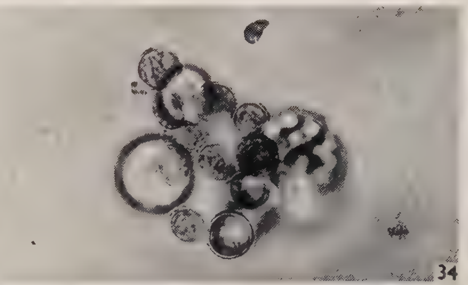
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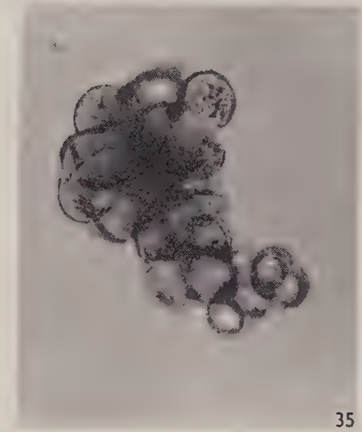
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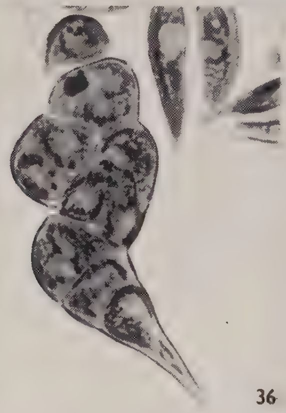
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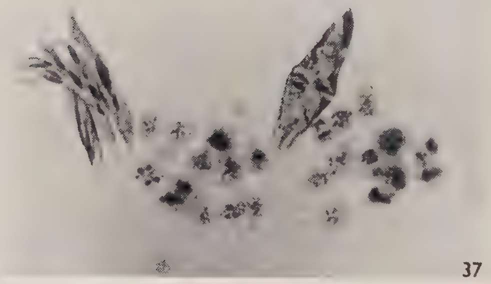
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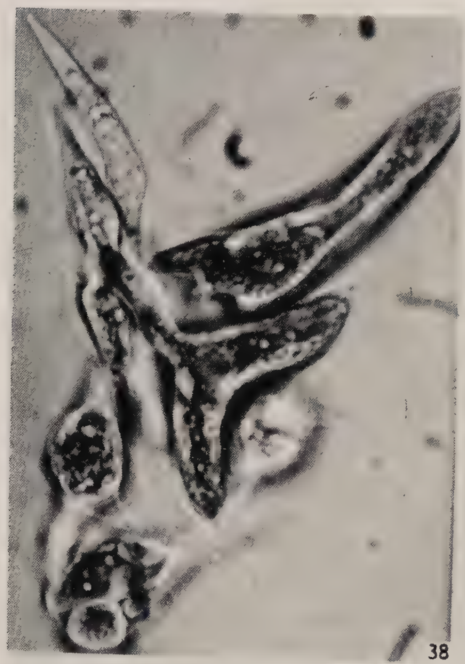

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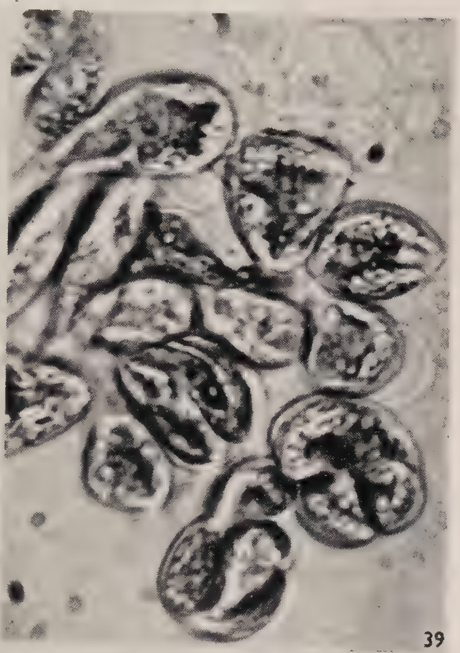

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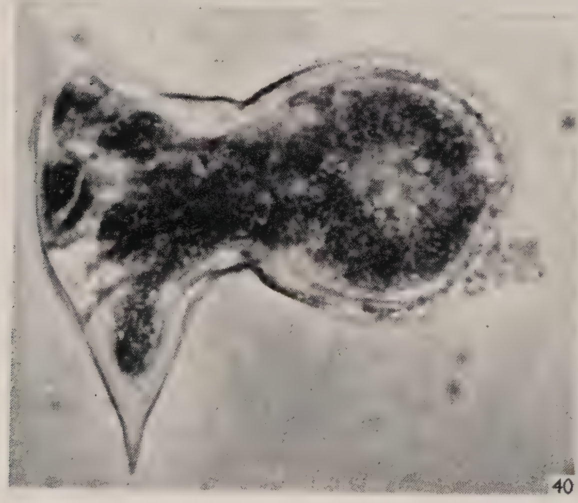

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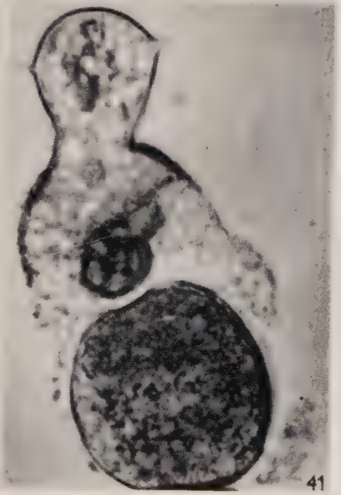

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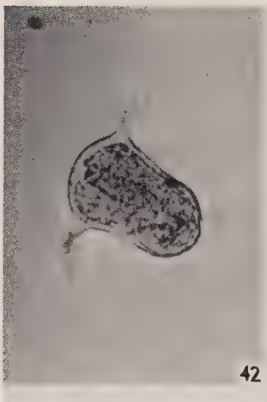
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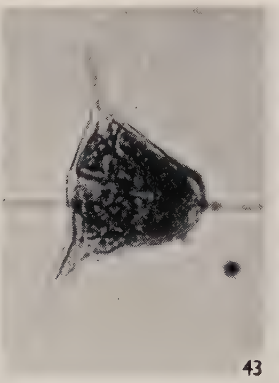
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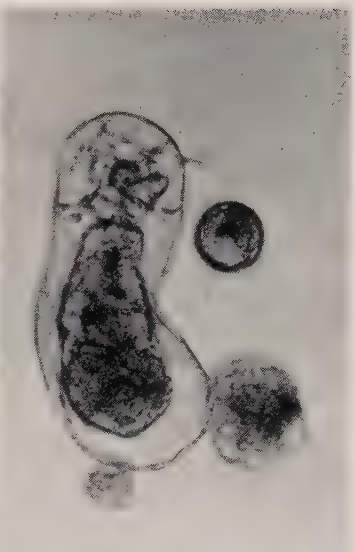
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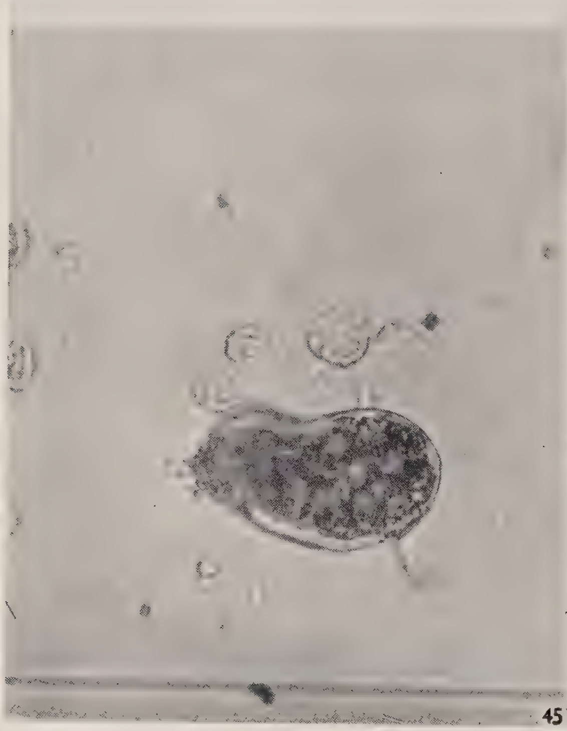
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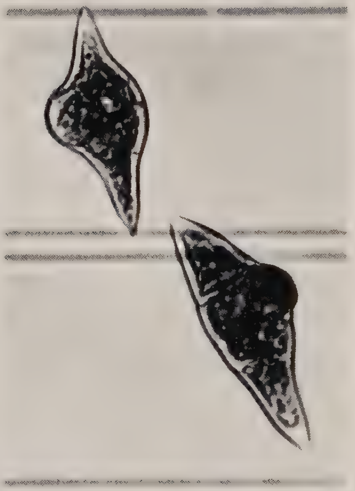
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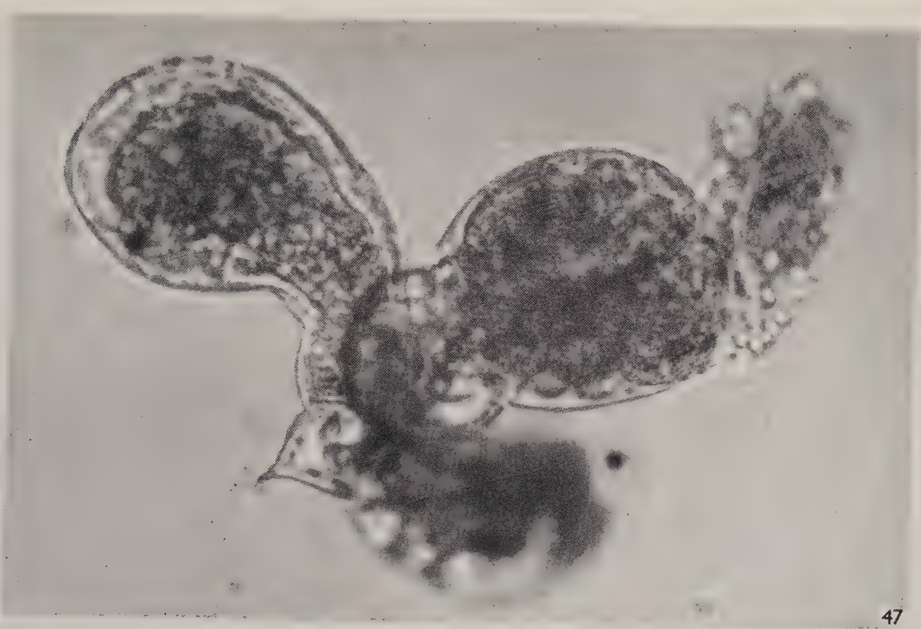
R. McMillan—Morphogenesis of ankistrodesmus, Plate 8



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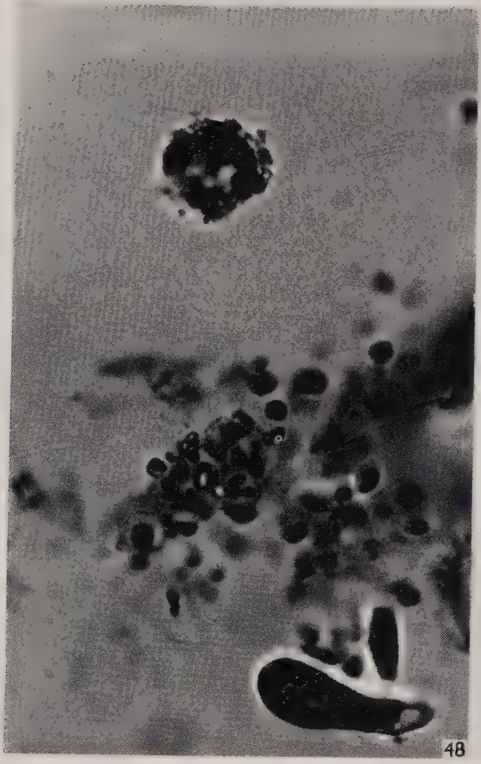

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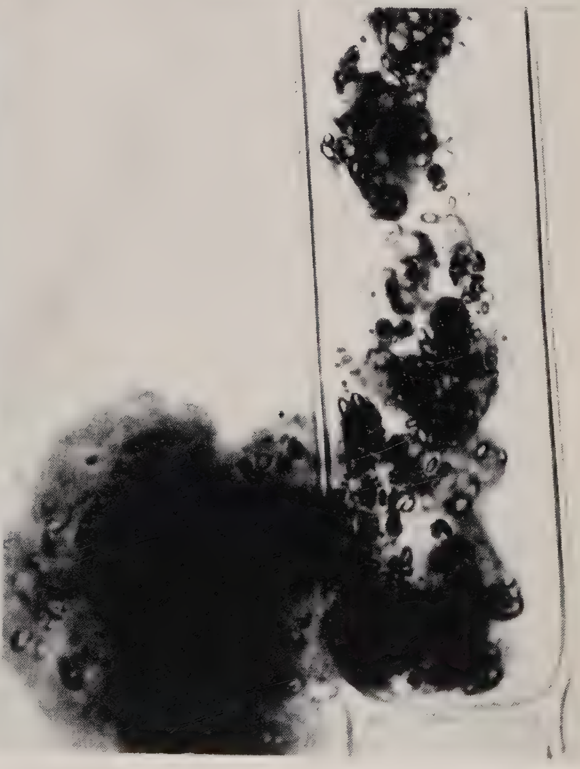

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R. McMillan—Morphogenesis of ankistrodesmus. Plate 9

CHODAT, R. (1909). Étude critique et expérimentale sur le polymorphisme des Algues. *Mém. publ. à l'occasion du Jubilé de l'Université de Genève.*

CHODAT, R. (1913). Monographie d'algues en culture pure. *Matér. Flore Cryptog. Suisse*, **4**, fasc. 2.

CHODAT, R. (1929). La mutation généralisée et les mutations chez le *Chlorella rubescens* Chodat. *C.R. Soc. Phys. Hist. nat. Genève*, **46**, 31.

CHODAT, R. & MALINESCO, O. (1893). Sur le polymorphisme du *Raphidium braunii* et du *Scenedesmus acutus* Corda. *Bull. Herb. Boissier*, I, **2**, 640.

CHU, S. P. (1942). The influence of the mineral composition of the medium on the growth of planktonic algae. I. Methods and culture media. *J. Ecol.* **30**, 284.

COHEN, S. S. & BARNER, H. D. (1956). Studies on the induction of thymine deficiency and on the effects of thymine and thymidine analogues in *Escherichia coli. J. Bact.* **71**, 588.

GERLOFF, G. C., FITZGERALD, G. P. & SKOOG, F. (1950). The isolation, purification, and nutrient solution requirements of blue-green algae. In *The Culturing of Algae*; a symposium, p. 27. Charles F. Kettering Foundation.

HOFFMAN-GROBÉTY, A. (1912). Contribution à l'étude des Algues unicellulaires en culture pure. I. *Raphidium minutum* Naeg. *Bull. Soc. Bot. Genève*, **4**, 73.

KLEBS, G. (1883). Über die Organisation einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien. *Untersuch. bot. Inst. Tübingen*, **1**, 233.

KLEBS, G. (1896). *Die Bedingungen der Fortpflanzung bei einigen Algen und Pilzen.* Jena: G. Fischer.

MCMILLAN, R. (1955). The effect of metabolites upon cellular development and morphology in certain Chlorococcales. Ph.D. dissertation, Northwestern University, Evanston, Illinois, U.S.A. (University Microfilms, Ann Arbor, Michigan, U.S.A.)

OETTLI, M. (1927). Recherches expérimentales sur cinq espèces élémentaires d'*Ankistrodesmus. Bull. Soc. bot. Genève*, **19**, 1.

NICKERSON, W. J. (1948). Enzymatic control of cell division in micro-organisms. *Nature, Lond.* **162**, 241.

NICKERSON, W. J. (1953). Enzymatic reactions in the cellular division mechanism of microorganisms. *Atti VI Congr. int. Microbiol.* **1**, 211.

NICKERSON, W. J. (1954). Experimental control of morphogenesis in microorganisms. *Ann. N.Y. Acad. Sci.* **60**, 50.

SCHRÖDER, B. (1954). Influence of various neutral salts on the growth, cell form and filament formation of *Stichococcus bacillaris* and several other green algae. *Arch. Mikrobiol.* **20**, 63.

VISCHER, W. (1920). Sur le polymorphisme de l'*Ankistrodesmus Braunii* (Naegeli) Collins. *Z. Hydrol.* **1**, 5.

WHITFIELD, J. F. & MURRAY, R. G. E. (1956). The effects of the ionic environment on the chromatin structures of bacteria. *Canad. J. Microbiol.* **2**, 245.

EXPLANATION OF PLATES

PLATE 1. Anatomy of *Ankistrodesmus braunii* from cultures in logarithmic phase, GFS medium. Magnification, ×900.

Fig. 1. Representative organisms after liberation from parent cell wall, showing old apical cap of mother, pyrenoids and typical chloroplast structure. Tube culture.

Fig. 2. Organism in slide culture immediately before cleavage.

Fig. 3. The same organism at same focal level five minutes later, showing shift in position of chloroplast.

Fig. 4. Negative print from Kodachrome transparency of cell at time of inoculation into slide culture, showing lack of apical secretion.

Fig. 5. Negative print from Kodachrome transparency of cell 15 min. after inoculation into slide culture, showing early apical secretion.

Fig. 6. Negative print from Kodachrome transparency of cell 2 hr. after inoculation into slide culture, showing secretion at both apices.
Fig. 7. Negative print from Kodachrome transparency of cell at second cleavage, showing growth and profuse apical secretion.

Plate 2. Developmental history of a single average cell of *Ankistrodesmus braunii* in slide culture, GFS medium. Magnification ×500, except fig. 13 which is ×220.

Fig. 8. Regeneration of chloroplast and apical secretion in a cell from stationary phase culture, 32 hr. after inoculation.
Fig. 9. First cleavage, 48 hr.
Fig. 10. Products of first division cycle, 78 hr.
Fig. 11. Growth of cells in fig. 10, 104 hr.
Fig. 12. Second division cycle, 110 hr.
Fig. 13. Scattering and aberrant forms, 12 days.

Plate 3. Cleavage and associated events in normal cells of *Ankistrodesmus braunii.* Magnification, ×600.

Fig. 14. Synchrony of cleavage in sister cells, during second division cycle, with initiation of cleavage at pyrenoid. Tube culture.
Fig. 15. Configuration of cells in 4-cell stage. Tube culture.
Fig. 16. Configuration of cells in 8-cell stage. Slide culture.
Fig. 17. Spiralling of daughter cells. Slide culture.
Fig. 18. Three-pronged and triangular sister cells from fourth division cycle. Tube culture.

Plate 4. Ageing and sporulation in *Ankistrodesmus braunii.* Magnification, various.

Fig. 19. Spiralling in a late division cycle with reduced elongation. 5 days, GFS medium. Magnification, ×350. Slide culture.
Fig. 20. Degeneration of chloroplast and early formation of oil droplets. Progeny of the cell traced in Pl. 2, 13 days, GFS medium, slide culture. Magnification, ×350.
Fig. 21. Division without elongation prior to sporulation, GFS medium, 7 days, slide culture. Magnification, ×350.
Fig. 22. Mature spore clusters, ensporulated single cell and vegetative cell, packed with oil droplets. GFS medium, 130 days, tube culture. Magnification, ×700.
Fig. 23. Spore cluster and spherical cells of 'Chlorella cycle' as described by Beijerinck; pigment and chloroplast structure normal. Yeast medium (medium no. 14), 130 days, tube culture. Magnification, ×700.
Fig. 24. Representative oil-packed vegetative cells of stationary phase. GFS medium, 22 days, slide culture. Magnification, ×350.

Plate 5. *Ankistrodesmus braunii*: exsporulation. Magnification, various.

Fig. 25. Cleavage, spiralling, escape, elongation and initiation of next division cycle in a single germinating spore cluster. GFS medium, tube culture. Magnification, ×550.
Fig. 26. Escape of protoplasts from spore-case prior to cleavage. GFS medium, slide culture. Magnification, ×550.
Fig. 27. Cleavage of escaped protoplasts in a group similar to that of fig. 26. GFS medium, slide culture. Magnification, ×275.
Fig. 28. A four-celled cluster with asynchronous development; growth and turgidity of intercalary cells have burst encasing the mother cell wall. GFS medium, slide culture, 6 hr. Magnification, ×275.
Fig. 29. Escape of first group of progeny from cluster of fig. 28, 78 hr. Magnification, ×275.
Fig. 30. Later division cycle and spiralling of first progeny; apical and right central cells pigmented. Magnification, ×275.
Fig. 31. A pisciform cell representing apical cell of a spore cluster, just prior to first cleavage. GFS medium, slide culture, 49 hr. Magnification, ×275.
Fig. 32. Progeny of cell in fig. 31, 78 hr. Magnification, ×275.

PLATE 6. *Ankistrodesmus braunii*: abnormal spore development; abortive 'zoospores'. Magnification, various.

Fig. 33. Large spore dividing (?) by constriction. Medium no. 14, tube culture, 130 days. Magnification, × 600.

Fig. 34. Group of double-walled, spherical cells. Medium no. 9, slide culture, 70 days. Magnification, × 300.

Fig. 35. *Protococcus*-like cluster. 4 % glucose (w/v) in GFS medium, slide culture, 28 days. Magnification, × 600.

Fig. 36. Growth of spore protoplasts without release. GFS medium, tube culture, 96 hr.

Fig. 37. Three sister cells, one in cleavage, one releasing normal progeny, and one releasing 'zoospores'. GFS medium + 5 × normal concentration of $[NO_3]^-$, slide culture, 48 hr. Magnification, × 300.

PLATE 7. Representative cell morphology of *Ankistrodesmus braunii* in varying concentrations of glucose. Magnification, × 750, except fig. 41, which is × 375.

Fig. 38. Representative cells in 0·04 % glucose (w/v). Tube culture, 5 days. Cells in 0·4 % (w/v) were similar in morphology.

Fig. 39. Representative cells in 2·0 % glucose (w/v). Tube culture, 5 days.

Fig. 40. Representative cell in 4 % glucose (w/v). Tube culture, 28 days.

Fig. 41. Another representative cell in 4 %, showing extreme of protoplast extrusion and flaky degeneration of cell wall. Slide culture, 28 days.

PLATE 8. Effect of high osmotic pressure upon morphogenesis of *Ankistrodesmus braunii*: developmental history of variant cells. Magnification, × 700.

Fig. 42. Beginning of equatorial bulge; incipient first cleavage furrow. 4 % glucose (w/v), slide culture, 72 hr.

Fig. 43. Slightly older cell. 4 % glucose (w/v), slide culture, 96 hr.

Fig. 44. Representative cell, 4 % glucose (w/v), slide culture, 28 days. One intact, spherical daughter cell (which later divided), two naked portions of protoplasmic ooze and a large protoplasmic bulge have formed. Most of the chambers formed by early cleavages in region of original cell dimensions have been emptied.

Fig. 45. Another cell from the same slide culture as the cell in fig. 44; 28 days. Widely scattered lipid material and a small amount of protoplasmic ooze have been liberated. Many small, intact, spherical cells are retained within the mother cell.

Fig. 46. Beginning of equatorial bulge. Penta-erythritol, saturated solution, slide culture, 28 days. Comparable stages occurred in glucose, 4 % (w/v) at 48–72 hr.

PLATE 9. Protoplasmic ooze and zoospore formation: comparison of *Ankistrodesmus* and *Cladophora*. Magnification, × 900.

Fig. 47. Cluster of sister cells of *A. braunii* (in part, out of focus). Lateral equatorial bulges, with protoplasmic extrusion from one cell. 4 % glucose (w/v), slide culture, 28 days.

Fig. 48. Extrusion of protoplasmic ooze in *Cladophora* sp., in a filament of freshly collected material which was producing zoospores abundantly. The internal, flexible protoplasmic membrane has formed a 'sausage' within the cell; compare figs. 40, 44, and 47.

Fig. 49. Extruded protoplasm, a zoospore and a germling, from the same *Cladophora* filament shown in Fig. 48. The print was over-developed to render the flagella of the zoospore visible. Compare fig. 37.

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di Menna, M. E. (1957). *J. gen. Microbiol.* **17**, 678–688

The Isolation of Yeasts from Soil

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SUMMARY: A series of comparative studies was made of soil yeasts in a yellow-brown earth originally under forest but now under pasture. Of six solid acid media used for primary isolations, an agar medium containing 4% (w/v) glucose and 1% (w/v) peptone gave the highest yeast counts. Two soil-extract agars and a modified Czapek-Dox agar gave slightly lower counts; a third soil-extract agar inhibited all but one yeast species and gave a much decreased count. The addition of a surface active agent to glucose peptone agar did not increase the count. Acid broth enrichment cultures gave a distorted picture of the species pattern shown by primary cultures on solid media. Prolonged mechanical shaking of soil dilutions before culturing, and the addition of a surface active agent to the diluent did not affect the yeast pattern seen, quantitatively or qualitatively.

Past experience in the isolation of yeasts from mixed microbial populations (Marples & di Menna, 1952; di Menna, 1945, 1955*a*, *b*, 1956) has shown that a modified Sabouraud agar (glucose 4% (w/v), peptone 1% (w/v)) brought to pH 4 is a useful medium for primary cultures. All but acidophilic species of bacteria are inhibited and usually the only microbial interference is by spreading moulds. However, this medium may have several defects, particularly for the isolation of yeasts from soil. The relatively high concentration of nutrients may give rise to a variety of microbial interactions on isolation plates. The high acidity and the lack of growth factors present in some soil extracts may interfere with the development of some species. A series of parallel quantitative and qualitative surveys of the soil yeasts of one area was made in order to compare the effectiveness of Sabouraud agar with a number of other media, and to find the most suitable pre-cultural treatment of soil dilutions.

METHODS

The soil studied was Korokoro silt loam from Judgeford, near Wellington, New Zealand. It was classified as a moderately leached, moderately weathered, yellow-brown earth derived from greywacke (Taylor, 1948). The original vegetation was mixed broadleaf forest which had been burnt off about a hundred years ago and replaced with browntop-sweet vernal pasture. The natural fertility is medium to low. The site sampled was a moderately sloping hilltop at an elevation of about 1000 ft. above sea level. It had never been ploughed and was grazed by sheep. Chemical analysis of a sample of the 0–6 in. layer was as follows: pH 5·3; P_2O_5 (1% citric acid soluble), 0·007%; total N %, 0·34; % base saturation, 36; C/N ratio, 16.

Rainfall of 50–60 in. is evenly distributed throughout the year. The average annual temperature is about 54° F. with a range of 46–61°.

Sampling methods. Samples were taken at a depth of 4 in. by thrusting a sterile container into the side of a freshly dug pit. Usually one sample was taken from each of five pits spaced along a 100-yard transect, but in May 1955 one sample was taken from each of two opposing sides of five pits. The first sampling pit (sample 1) was always dug at the lower and wetter end of the slope. Forest dimples were avoided, pits being dug in areas of even slope. Collections of soil were made in May and October 1955, and in February, May, August and October 1956.

In addition, samples of pasture leaves were collected in May, August and October 1956 so that their yeast flora could be examined for control purposes. In May a single composite sample was taken; in August two samples were collected, one of grass leaves and one of clover leaves, and in October five samples were taken, one from directly above each of the five sample pits from which soils were collected. The leaves were clipped with sterile scissors into sterile containers. Care was taken to avoid gathering material splashed with soil or contaminated with earthworm castings, but when the pasture was closely grazed this was difficult.

Turves from each of the five sample pits dug in October 1956 were taken back to the laboratory so that grass roots could be dissected from them for culturing.

Cultivation methods. All soil samples and the washings from roots and leaves were cultured on plates of modified Sabouraud agar (4 %, w/v, glucose; 1 %, w/v peptone, British Drug Houses Ltd.; 2 %, w/v, Davis' New Zealand agar; referred to henceforth as pH 4 glucose peptone agar) which had been brought to pH 4 by the addition of N-HCl to the molten medium immediately before pouring. The poured plates were dried for a short time in a warm oven so that the medium would absorb the moisture of the inoculum.

The soil dilution and culturing technique routinely used was as follows. Ten g. soil from each sample were weighed out with sterile precautions and made into serial dilutions of 1/250, 1/500 and 1/1000 with sterile tap water. The soil dilutions were shaken by hand for about 30 sec. as each dilution was made and were reshaken briefly before inocula were removed. An inoculum of 0·5 ml. was put on each 10 cm. plate and spread with a glass spreader. Plates were incubated upside down at room temperature.

Leaf samples and roots dissected from turves were shaken with sterile tap water and the washings spread on pH 4 glucose peptone agar plates. All samples were cultured as soon as possible after collection; any held overnight were kept in a refrigerator.

Cultures were incubated for 4–6 days before examination, the time varying with the season, the medium used, and the number of moulds appearing on the plates. Yeasts to be identified were picked systematically from the plates and subcultured into yeast extract glucose broth (pH 4). The number of isolates identified per sample per treatment varied from 10 to 50.

Cultures were identified by the criteria of Lodder & Kreger-van Rij (1952). *Pullularia pullulans* (De Bary & Low) Berkhout was treated as a yeast.

Parallel cultures. Five other solid media were tested against pH 4 glucose peptone agar. Three of these were soil-extract agars and two were media chosen particularly for their ability to decrease mould colony size. The first of these was the modified Czapek-Dox agar used by Neill & Armstrong (1955) in their survey of blind seed disease, sucrose as the carbon source being replaced by Marmite. The second medium was glucose peptone agar to which had been added 0·01 %, w/v, Lubrol W (Imperial Chemical Industries Ltd.), a non-ionic surface active agent which gives a neutral heat-stable solution.

The soil-extract agars were prepared by autoclaving one part of soil from the A horizon with three parts of tap water for 30 min. at 15 lb./sq.in. (121°), filtering, diluting the filtrate by half with tap water, and adding sufficient agar to set. The three soils used were: Taita silt loam, a similar soil to Korokoro but developed under beech forest; Cass fine sandy loam, a strongly leached, weakly weathered, yellow-brown earth from greywacke alluvium; Waiwhetu silt loam, a recent soil from greywacke alluvium.

All these media were used at pH 4 and were inoculated from the same soil dilutions as used for the cultures on pH 4 glucose peptone agar.

Broth cultures. Some acid broth 'enrichment cultures' were made, rather so that results could be compared with those of other workers (Starkey & Henrici, 1927; Capriotti, 1955) than because enrichment was necessary or desirable. Three acid broths were used: glucose yeast extract broth (Kurung, 1942), 1 % peptone water, and carrot infusion. The broths were brought to pH 4, dispensed in 90 ml. lots in 250 ml. Erlenmeyer flasks, and sterilized at 121° for 15 min. Ten g. soil from each sample examined were inoculated into a flask of each kind of broth and incubated at room temperature for a week, at the end of which time a heavy pellicle of moulds had formed. Samples of broth from beneath the pellicles were removed with Pasteur pipettes and plated out on pH 4 glucose peptone agar. Recovery of ten yeasts/sample/treatment was attempted, but this was not always possible.

Pre-culturing treatments. Yeast colonies which developed on plates inoculated with soil suspensions may have arisen from single organisms or from aggregates. Skinner (1951) reported that by use of a shaking technique it was possible to determine whether actinomycetes in soils occurred predominantly as spores or as vegetative particles. It was hoped that the use of a similar shaking method would give information upon the state of yeasts in soil. Soil samples from the August 1956 collection were diluted serially with sterile tap water and cultured by the standard method on pH 4 glucose peptone agar. The dilutions remaining after the inocula had been removed were then shaken for 15 min. in a mechanical reciprocal shaker at 300 strokes/min. with an amplitude of 0·5 in. Cultures were then made from the mechanically-shaken dilutions. A parallel series of dilutions from the same collection were made in tap water containing 0·01 %, w/v, Lubrol W and were cultured in a similar manner, before and after 15 min. mechanical shaking.

RESULTS

On pH 4 *glucose peptone agar*

There was very great site-to-site variation in numbers of soil yeasts. Counts from single samples varied between 6000 and 240,000 yeast colonies/g. soil. No seasonal trend in numbers was distinguishable. Four species, *Cryptococcus albidus* (Saito) Skinner, *Cryptococcus terreus* di Menna, *Candida curvata* (Diddens & Lodder) Lodder & van Rij and *Schizoblastosporion starkeyi-henricii* Ciferri occurred in important numbers; very few strains of other species were recovered. Of these four species *Cryptococcus terreus* was the commonest in most collections. *Schizoblastosporion starkeyi-henricii* made up 75 % of the isolates from the three higher and drier sites sampled in May 1955, but was represented in only small numbers in the five other soil collections. Detailed results are given in Table 1.

Yeasts on pasture leaves showed a seasonal change of species pattern (Table 2). In May (autumn) *Sporobolomyces odorus* Derx, *Candida humicola* (Daszewska) Diddens & Lodder, and *Schizoblastosporion starkeyi-henricii* were isolated from the washings, but none of these species was present in the August and October (late winter and spring) collections, when *Cryptococcus laurentii* (Kafferath) Skinner and *Torulopsis aeria* (Saito) Lodder were dominant. The species pattern of yeasts in root washings was similar to that in soil samples collected at the same time.

Comparative cultures on solid media

Yeast counts on the three soil-extract agars were smaller than counts of the same samples cultured on pH 4 glucose peptone agar (Table 3). The species pattern given by cultures on Taita and Cass soil-extract agars were similar to those given by pH 4 glucose peptone agar, except that proportions of *Cryptococcus terreus* and *Schizoblastosporion starkeyi-henricii* were decreased a little. The count on Waiwhetu soil-extract agar was only a quarter of that on pH 4 glucose peptone agar and *Candida curvata* was the only yeast recovered from the cultures.

Results from the two series of isolations made on Marmite + Czapek-Dox agar were similar to those made on the Cass and Taita soil-extract agars; counts were lower than on pH 4 glucose peptone agar and the proportions of *Cryptococcus terreus* and *Schizoblastosporion starkeyi-henricii* were slightly diminished. The count on glucose peptone agar with Lubrol W was only three-quarters of that on pH 4 glucose peptone agar without additions and no decrease in mould colony size was seen.

Enrichment cultures in acid broths

No yeasts were recovered from the carrot infusion cultures, spreading moulds completely covering the plates. Similarly, no yeasts were recovered from one of the soil samples incubated in glucose yeast extract broth; in other instances it was not possible to recover ten yeasts/sample/treatment. *Candida humicola*,

Table 1. *Yeasts from Korokoro soil isolated on* pH 4 *glucose peptone agar*

	Debaryomyces kloeckeri	*Debaryomyces nicotianae*	*Cryptococcus laurentii*	*Cryptococcus albidus*	*Cryptococcus luteolus*	*Cryptococcus diffluens*	*Cryptococcus terreus*	*Candida humicola*	*Candida curvata*	*Trichosporon cutaneum*	*Schizoblastosporion starkeyi-henricii*	*Rhodotorula glutinis*	*Rhodotorula mucilaginosa*	Total	Yeast colonies (thousand)/g. soil
May 1955															
Site 1	.	.	.	15	1	.	29	.	30	4	.	.	1	80	.
2	.	.	.	12	.	3	46	.	7	2	10	.	.	80	.
3	.	.	.	2	.	.	9	.	2	.	67	.	.	80	.
4	1	1	–	5	.	.	3	.	7	3	59	1	.	80	.
5	.	.	.	.	.	.	8	3	3	5	61	.	.	80	.
Total	**1**	**1**	.	**34**	**1**	**3**	**95**	**3**	**49**	**14**	**197**	**1**	**1**	**400**	.
Oct. 1955															
Pooled	.	.	.	3	.	.	31	.	6	.	10	.	.	50	200
February 1956															
Site 1	.	.	.	.	.	.	6	.	4	.	.	.	.	10	90
2	.	.	.	.	.	.	5	.	5	.	.	.	.	10	14
3	.	.	.	1	.	.	7	.	2	.	.	.	.	10	46
4	.	.	.	.	.	.	6	.	.	.	4	.	.	10	73
5	.	.	.	.	.	.	5	.	4	.	1	.	.	10	47
Total	.	.	.	**1**	.	.	**29**	.	**15**	.	**5**	.	.	**50**	.
May 1956															
Site 1	.	.	.	7	.	.	11	.	1	1	.	.	.	20	72
2	.	.	.	3	.	.	5	.	9	.	3	.	.	20	45
3	.	.	.	9	.	.	8	.	3	.	.	.	.	20	188
4	.	.	.	5	.	.	4	.	11	.	.	.	.	20	55
5	.	.	.	12	.	.	2	.	5	.	1	.	.	20	96
Total	.	.	.	**36**	.	.	**30**	.	**29**	**1**	**4**	.	.	**100**	.
August 1956															
Site 1	.	.	.	1	.	.	2	.	17	.	.	.	.	20	27
2	.	.	1	.	.	.	15	1	.	1	2	.	.	20	73
3	.	.	.	3	.	.	10	.	7	.	.	.	.	20	115
4	.	.	.	3	.	.	9	.	8	.	.	.	.	20	6
5	.	.	.	4	.	.	10	.	6	.	.	.	.	20	155
Total	.	.	**1**	**11**	.	.	**46**	**1**	**38**	**1**	**2**	.	.	**100**	.
October 1956															
Site 1	.	.	.	2	.	.	13	.	2	1	2	.	.	20	105
2	.	.	.	2	.	.	7	.	9	1	1	.	.	20	35
3	.	.	.	4	.	.	1	.	15	.	.	.	.	20	108
4	.	.	.	4	.	.	10	.	4	.	2	.	.	20	113
5	.	.	.	4	.	.	11	.	3	.	2	.	.	20	97
Total	.	.	.	**16**	.	.	**42**	.	**33**	**2**	**7**	.	.	**100**	.

Table 2. *Yeasts from the washings of the leaves and roots of pasture grasses and herbs cultured on* pH 4 *glucose peptone agar*

	Sporobolomyces sp.	*Cryptococcus laurentii*	*Cryptococcus albidus*	*Cryptococcus terreus*	*Torulopsis aeria*	*Candida humicola*	*Candida curvata*	*Trichosporon cutaneum*	*Schizoblastosporion starkeyi-henricii*	*Rhodotorula mucilaginosa*	*Rhodotorula flava*	*Rhodotorula* sp.	*Pullularia pullulans*	Total
May 1956														
Pasture leaves	1	.	.	.	.	5	.	.	5	.	.	9	.	20
August 1956														
Grass leaves	.	.	.	.	18	.	.	.	.	1	.	.	1	20
Clover leaves	.	6	.	.	12	.	.	.	.	1	1	.	.	20
October 1956														
Pasture leaves	.	47	2	2	43	.	.	.	.	3	2	1	.	100
Grass roots	.	.	6	57	3	2	13	1	18	.	.	.	.	100

Table 3. *Yeasts isolated from Korokoro soil on different solid acid media*

	Cryptococcus albidus	*Cryptococcus terreus*	*Candida curvata*	*Trichosporon cutaneum*	*Schizoblastosporion starkeyi-henricii*	Total	Yeasts per gram in thousands Sample 1	2	3	4	5
October 1955											
On pH 4 glucose peptone agar	3	31	6	.	10	50	200 (pooled sample)				
On Taita soil extract agar	10	21	15	.	4	50	120 (pooled sample)				
On pH 4 glucose peptone agar with Lubrol W	2	41	1	.	6	50	145 (pooled sample)				
February 1956											
On pH 4 glucose peptone agar	1	29	15	.	5	50	90	14	46	73	47
On Cass soil extract agar	10	17	20	.	3	50	90	13·5	49	27	29
On Waiwhetu soil extract agar	.	.	50	.	.	50	8	8·5	21	15	8·5
On Marmite agar	8	24	15	.	3	50	85	10	56	40	51
May 1956											
On pH 4 glucose peptone agar	36	30	29	1	4	100	72	45	188	55	96
On Marmite agar	23	21	55	.	1	100	63	31	126	59	93

Table 4. *Yeasts recovered from cultures of soils in acid broths, collection of February* 1956

	Rhodotorula sp.	*Cryptococcus terreus*	*Candida humicola*	*Candida curvata*	*Schizoblastosporion starkeyi-henricii*	Total
From glucose yeast extract broth	.	4	22	7	7	40
From peptone water	5	9	23	.	11	48
From carrot infusion			No yeasts recoverable			

Table 5. *Effects of pre-cultivation treatments of soil dilutions by* 15 *min. mechanical shaking and by a surface active agent. Collection of August* 1956

											Yeasts per gram in thousands				
Precultural treatment	*Cryptococcus laurentii*	*Cryptococcus albidus*	*Cryptococcus terreus*	*Torulopsis aeria*	*Candida humicola*	*Candida curvata*	*Trichosporon cutaneum*	*Schizoblastosporion starkeyi-henricii*	*Rhodotorula marina*	Total	Sample 1	2	3	4	5
1. Standard method	1	11	46	.	1	38	1	2	.	100	27	73	115	6	155
2. Mechanical shaking	1	20	43	.	1	30	.	5	.	100	14	79	127	6	135
3. Surface active agent	.	17	40	.	.	36	.	6	1	100	21	77	140	9	244
4. Mechanical shaking and surface active agent	.	14	48	1	.	31	.	6	.	100	19	68	80	11	181

Cultures were made on pH 4 glucose peptone agar. The same dilutions were used for treatments 1, and 2 which are strictly comparable. Another series of dilutions from the same soil samples were used for treatments 3 and 4.

which was not isolated when the samples were cultivated on solid media, was the most common species in both the glucose yeast extract broth and the peptone water cultures. The results are given in Table 4.

Pre-culturing treatments

The counts obtained before and after 15 min. shaking of soil dilutions in sterile water were similar. There was some decrease in counts after mechanical shaking in 0·01 %, w/v Lubrol W in tap water, but this may have been experimental error rather than a real effect. Results are given in Table 5.

DISCUSSION

Of the six media used pH 4 glucose peptone agar consistently gave the highest yeast counts and so is most likely to reflect accurately the species pattern in soil. Any benefit from decrease in size of mould colonies by the use of starvation media such as Marmite + Czapek-Dox agar or soil-extract agars was more than offset by decreases in the numbers of yeast colonies which developed. The failure of a surface active agent to decrease mould colony size was probably due to the inoculum being spread on the surface of the medium and leaving a film of moisture. Unfortunately pour plates are not suitable for the isolation and enumeration of yeasts, for the colonies do not usually grow to the surface of the medium, which must therefore be dissected if they are to be subcultured. Also it is often necessary when counting to examine colonies with a low-power microscope objective in order to distinguish between yeasts and bacteria, and this is better done when the growth is on the surface.

Soil extract does not seem to be necessary for the growth of soil yeasts. Indeed the use of soil-extract agars appears to be a practice which can give misleading results. Results of surveys made using Cass and Taita soil-extract agars were very similar to those made using Marmite agar, which suggests that while these soil extracts did not stimulate soil yeasts and had a low nutrient value, they were able to support yeast growth tolerably. On the other hand, when Waiwhetu soil-extract was used the count was much decreased and only *Candida curvata* was recovered from the cultures. It is possible that the Waiwhetu soil extract contained a heat-stable antibiotic, or toxic material produced by autoclaving, to which *Candida curvata* was the only member of the yeast flora that was insensitive. Dobbs & Hinson (1953) reported a fungistatic agent, widespread in soils, which may be removed by severe heating or prolonged desiccation, and the effect of which may be masked by the addition of glucose or other nutrients.

When 1 %, w/v, British Drug Houses Ltd. peptone and 0·5 %, w/v glucose were added to some of the original batch of Waiwhetu soil-extract agar, subcultures of strains of *Rhodotorula* sp., *Cryptococcus albidus*, *Cryptococcus terreus*, *Candida curvata* and *Schizoblastosporion starkeyi-henricii*, isolated from Korokoro soil and grasses, grew as well on it as on control cultures of pH 4 glucose peptone agar without soil extract. The fungistatic agent of Dobbs & Hinson (1953) may account for the properties of the Waiwhetu soil extract, although it would be necessary to presume that the same agent was lacking or in ineffective concentrations in the Cass and Taita soil extracts. It is still possible that a soil extract added to pH 4 glucose peptone agar may foster the growth of some exacting species which would not appear on the more simple nutrient medium.

Broth enrichment cultures. These cultures, which yielded no information about yeast numbers in the soils, gave a distorted picture of the species pattern disclosed by cultures on solid media. *Cryptococcus terreus* and *Candida curvata* which were dominant in cultures on solid media were recovered from the broth cultures but in only minor proportions. They were replaced in order of frequency by *Candida humicola*, a species of which very few isolates have been recovered from Korokoro soil but which was common on grass leaves in the autumn. An acid broth which is rich in nutrients that are lacking or in very low concentrations in the soil, and which is overrun by zymogenous moulds, is a microcosm in which it is unlikely that yeasts suited for growth in the soil will flourish.

Pre-cultivation treatment of soil dilutions. Since mechanical shaking of soil dilutions, with or without the addition of a surface active agent, had no consistent effect upon the yeast count, it seems that naturally-occurring yeast colonies in the soil are either loosely held together and break apart when the soil is made into a slurry, or else are very stable and cannot be disturbed by prolonged agitation or alteration of the surface tension.

Relationship between yeast floras of phyllosphere and soil. With the exception of *Schizoblastosporion starkeyi-henricii* no yeast species occurred commonly both on leaves and in soil. *Cryptococcus terreus* was the most frequently occurring yeast in most soil collections, although *Cryptococcus albidus*, *Candida curvata* and *Schizoblastosporion starkeyi-henricii* were all dominant on occasion. Site variation in species pattern was not usual in natural tussock grassland soils, neither under the original vegetation nor under introduced pasture (di Menna, unpublished), and variation in the Korokoro soil may indicate a micropopulation still transitional between forest and grassland types. Site variation in numbers in Korokoro soil was also much greater than in natural grassland soils and may possibly be related to the discontinuous effect of rotted forest remains in an unploughed soil. A seasonal change in numbers may have been obscured by the site variation, but it is also likely that no great seasonal fluctuation occurs in an area where the distribution of rainfall is even and the temperature range throughout the year is small.

Ruinen (1956) noted a limited and characteristic yeast flora on the phyllosphere of tropical plants, and Last (1955) observed seasonal variation in numbers of yeasts on the phyllosphere of cereal crops. *Cryptococcus laurentii* and *Torulopsis aeria*, which were the commonest isolates from washings of late winter and spring leaf samples, were not found on leaves taken in the autumn, and were isolated from the soil in very small numbers and only in the August and October collections. On the other hand, *Schizoblastosporion starkeyi-henricii*, present on the pasture leaves in the autumn, was the predominant yeast in the May 1955 soil collection and occurred consistently, although in small proportions, in all other soil collections. The ecological position of this species is difficult to determine; physiologically it differs from the yeasts which seem to be true soil inhabitants. *S. starkeyi-henricii* does not use nitrate nitrogen, is uncapsulated, and cannot synthesize growth factors or starch (di Menna, 1955*a*). It is tempting to suggest that it occurred in unusually large numbers on pasture leaves in the autumn of 1955, and that the large proportion of its colonies on plates inoculated with soil collected at that time had grown from inactive organisms washed into the soil. Nevertheless, it was able to maintain itself and was recovered regularly in small numbers from all soil collections, including those taken in the spring, when it did not occur on leaves. Also, Lund (1954) recovered it from European soils, and Starkey & Henrici (1927) found one strain in a North American soil sample.

It seems desirable therefore, that an examination of the phyllosphere yeast flora be made in parallel with soil yeast studies, at least until more is known of the amount of interchange between the two floras, and particularly where the micropopulation may be in a disturbed state. At present a relatively rich, solid, acid medium appears to be best for the isolation of yeasts from soil. Although it encourages the growth of spreading moulds, it also stimulates yeast multiplication sufficiently to compensate for this.

My thanks are due to Mr L. C. Blakemore of the Soil Bureau for the chemical analysis of Korokoro soil, and to other colleagues in the Bureau for assistance in the selection of a sampling site. Soil Bureau Publication No. 119.

REFERENCES

Capriotti, A. (1955). Yeasts in some Netherlands soils. *Leeuwenhoek ned Tijdschr.* **21**, 145.

Dobbs, C. G. & Hinson, W. H. (1953). A widespread fungistasis in soils. *Nature, Lond.* **172**, 197.

Kurung, J. M. (1942). The isolation and identification of pathogenic fungi from sputum. *Amer. Rev. Tuberc.* **46**, 365.

Last, F. T. (1955). Seasonal incidence of *Sporobolomyces* on cereal leaves. *Trans. Brit. mycol. Soc.* **38**, 221.

Lodder, J. & Kreger-van Rij, N. J. W. (1952). *The Yeasts. A Taxonomic Study.* Amsterdam: North Holland Publishing Co.

Lund, A. (1954). *Studies on the Ecology of Yeasts.* Copenhagen: Munksgaard.

Marples, M. J. & di Menna, M. E. (1952). The incidence of *Candida albicans* in Dunedin, New Zealand. *J. Path. Bact.* **64**, 497.

Menna, M. E. di (1954). Non-pathogenic yeasts of the human skin and alimentary tract. *J. Path. Bact.* **68**, 89.

Menna, M. E. di (1955*a*). A search for pathogenic species of yeasts in New Zealand soils. *J. gen. Microbiol.* **12**, 54.

Menna, M. E. di (1955*b*). A quantitative study of air-borne fungus spores in Dunedin, New Zealand. *Trans. Brit. mycol. Soc.* **38**, 119.

Menna, M. E. di (1956). A search for *Cryptococcus neoformans* in milk. *Leeuwenhoek ned Tijdschr.* **22**, 331.

Neill, J. C. & Armstrong, C. S. (1955). An aerial survey of ascospore distribution of blind-seed disease of ryegrass (*Gloeotinia* (*Phialea*) *temulenta*). *N.Z. J. Sci. Tech.* A. **37**, 106.

Ruinen, J. (1956). Occurrence of *Beijerinckia* species in the 'phyllosphere'. *Nature, Lond.* **177**, 220.

Skinner, F. A. (1951). A method for distinguishing between viable spores and mycelial fragments of Actinomycetes in soils. *J. gen. Microbiol.* **5**, 159.

Starkey, R. L. & Henrici, A. T. (1927). The occurrence of yeasts in soils. *Soil Sci.* **23**, 33.

Taylor, N. H. (1948). Soil Map of New Zealand. *N.Z. Soil Bur. Map. S.B.* no. 280.

(*Received* 14 *June* 1957)

NICHOLAS, D. J. D. (1957). *J. gen. Microbiol.* **17**, 689–698

The Effect of Molybdenum Deficiency on the Catalase and Peroxidase Content of *Neurospora crassa*

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SUMMARY: Catalase and peroxidase were markedly decreased in quantity in molybdenum-deficient felts of *Neurospora crassa*. Addition of molybdenum aseptically and *in vivo* to mats deficient in molybdenum restored the activities of the two enzymes and the yield of mycelium to the amounts in the control treatments. Infiltration of hydrogen peroxide to growing felts, deficient in molybdenum, after 3 days of growth, resulted in a partial reconstitution of catalase to 18 % and peroxidase to 50 % of the control levels after a further 2 days growth but the weight of mycelium remained unchanged. There was no positive correlation between the activity of either enzyme at various stages of purification and its molybdenum content as determined by a radioassay method. The purified enzymes were not activated by molybdenum. The effect of molybdenum deficiency on the two iron-containing enzymes is probably indirect, resulting from a decrease in the activity of molybdenum-dependent flavoproteins which produce hydrogen peroxide, the common substrate for catalase and peroxidase.

In recent years molybdenum has been shown to be essential for the metabolism of some micro-organisms, higher plants and animals. Molybdenum is a constituent of nitrate reductase found in some bacteria (Nicholas & Nason, 1954*c*), in fungi (Nicholas, Nason & McElroy, 1954; Nicholas & Nason, 1954*a, b*), and in green plants (Nicholas & Nason, 1955). During the enzymic reduction of nitrate, molybdenum undergoes a valency change involving Mo^{6+} and Mo^{5+} (Nicholas & Stevens, 1955). When ammonium ion is the only source of nitrogen, however, nitrate reductase does not usually form in plants and the molybdenum requirement, although considerably decreased, is not completely eliminated. Thus molybdenum appears to be required for metabolic processes other than for the reduction of nitrate. The present paper amplifies a preliminary publication in which it was shown that a deficiency of molybdenum in *Neurospora crassa*, wild type 146, decreased the amounts of catalase and peroxidase obtained in cell-free extracts of the mycelial felts (Nicholas, 1956). It is also indicated that the decrease in amount of the two iron-containing enzymes is probably caused by a diminution of hydrogen peroxide production in Mo-deficient felts.

METHODS

Organism. Neurospora crassa, microconidial, wild type 146, was kindly supplied by Dr William D. McElroy (McCollum-Pratt Institute, Johns Hopkins University, Baltimore, U.S.A.). The fungus maintained at 30° on nutrient agar slopes (20 g. agar, 5 g. yeast extract, 5 g. malt extract/litre of culture medium) was subcultured weekly to ensure that it made vigorous growth.

Culture medium. The fungus was grown in the following basal liquid medium: sucrose, 20 g.; NH_4NO_3, 2 g.; Na tartrate, 1 g.; KH_2PO_4, 3 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 0·1 g.; $CaCl_2$, 0·1 g.; biotin, 5 μg.; $FeCl_3.6H_2O$, $9{\cdot}6 \times 10^{-4}$ g.; $ZnSO_4.7H_2O$, $8{\cdot}8 \times 10^{-3}$ g.; $CuCl_2.2H_2O$, $2{\cdot}7 \times 10^{-4}$ g.; $MnCl_2.4H_2O$, $7{\cdot}2 \times 10^{-5}$ g.; $Na_2MoO_4.2H_2O$, $1{\cdot}2 \times 10^{-5}$ g.; glass-distilled water to 1 l.; pH 4·8.

Removal of molybdenum from the basal medium. Molybdenum was effectively removed from a solution of the macronutrients including sucrose adjusted to pH 2·0 with 6N-HCl, by the copper sulphide co-precipitation method (Nicholas & Fielding, 1951; Nicholas, 1952). The pH was then re-adjusted to 4·8 with pure NaOH, prepared by passing 5 % (w/v) NaOH through an Amberlite column IRA 400 (OH), (3 ft. by 2 in.) which was well washed with glass-distilled water before use. The micronutrient elements which were added to the media after the purification were spectrographically pure reagents obtained from Johnson and Matthey, Hatton Garden, London, E.C. 1. Biotin was obtained in ampoules containing 25 μg. biotin from the Ashe Laboratories (Leatherhead, Surrey). The medium was dispensed in 200 ml. amounts in 1 l. Erlenmeyer flasks, inoculated with a suspension of the fungus mycelium contained in sterile glass-distilled water, and incubated in the dark at 28°. The cultures were shaken daily to prevent sporulation of the fungus.

Preparation of cell-free extracts. The mycelial mats from normal and Mo-deficient cultures, collected separately in Buchner funnels, and washed well in glass-distilled water, were frozen for 3 hr. at −17°. They were then ground in a mortar with three times their weight of cold 0·1 M-phosphate buffer (pH 7·5) and then in a Ten Broeck glass macerater (made to specification by Jencons Ltd., Hemel Hempstead, Hertfordshire) at 0°. The solutions were centrifuged at 2000 ***g*** for 10 min. at 4°. Over 90 % of each of the two enzymes was present in the supernatant solutions.

Enzyme assays. Catalase was determined in samples of the extracts by the perborate method (Feinstein, 1949); the enzyme activity being expressed as μmole perborate degraded/5 min./mg. protein.

Peroxidase was assayed by the method of Smith, Robinson & Stotz (1949) as the change in log I_0/I of 0·001/min., calculated from 15 to 75 sec./mg. protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Radioassay of molybdenum-99 in enzyme fractions. Carrier-free ^{99}Mo, as sodium molybdate, was obtained from A.E.R.E. Harwell. Ordinary sodium molybdate (A.R.) was added to the tracer so that 20 μg. Mo was labelled with 4 μC ^{99}Mo/l. culture solution. The culture solutions were first freed from Mo by the method of Nicholas (1952) and then inoculated with a suspension of the fungus mycelium. After 3 days of growth, 0·8 μC ^{99}Mo/4 μg Mo as sodium molybdate contained in 5 ml. glass-distilled water, was added aseptically to each flask containing 200 ml. culture medium. After a further 2 days of incubation the felts were harvested and the enzymes extracted as described above. The two enzymes were fractionated by ammonium sulphate precipitation, carbon adsorption at 4° and by ethanol precipitation at $-15°$. The protein fractions, dispensed in 0·1 M-phosphate buffer (pH 7·5) were radio-assayed in an M6 type tube (Veal 16). Isotopic decay and minor scale fluctuations were allowed for by counting a sample of the standard solution used for the treatments after assay of every three or four experimental solutions.

RESULTS

Effect of molybdenum deficiency

The data in Fig. 1 show the effects of molybdenum deficiency on the content of the two iron-containing enzymes in the mycelial felts harvested after 3, 4 and 5 days of growth. The deficiencies of molybdenum in Expts. A, B and C resulted in yields of 35, 50 and 65 % of the weight of the normal non-deficient felts on the 5th day on growth.

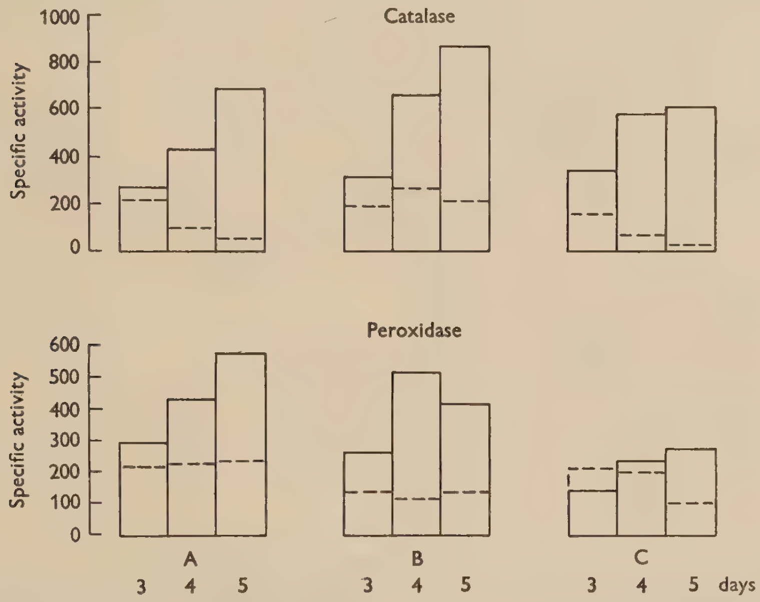

Fig. 1. The effect of the degree of Mo-deficiency on the content of catalase and peroxidase in *Neurospora crassa* after 3, 4 and 5 days of growth. In A, B and C, each subunit comprises mean values for catalase or peroxidase obtained in three independent experiments. Yields of approximately 35, 50 and 65 % of that of normal felts were obtained on the 5th day in A, B and C respectively. ——, catalase or peroxidase from normal felts; - - - -, catalase or peroxidase from Mo-deficient felts.

Catalase production increased in the normal felts from the 3rd to the 5th day and the amount of peroxidase was usually greater on the 4th and 5th days than on the 3rd day. The effect of molybdenum in decreasing catalase and peroxidase activity was more pronounced at the 4th and 5th days. In Expt. C, where the deficiency was less severe, catalase production was markedly decreased at the three stages of growth, whereas peroxidase was decreased on the 5th day only. Thus a deficiency of molybdenum seems to decrease production of catalase before that of peroxidase.

The fungus was grown with various quantities of Mo and the amounts of the two enzymes were determined in extracts of the felts; the results are illustrated in Fig. 2. An increase in the molybdenum content of the medium from 0 to 4 μg./200 ml. basal solution was paralleled by increased production of the two enzymes in the felts. The maximum activities were reached with 4 μg. Mo/200 ml. basal solution; the addition of 8, 16 and 32 μg. depressed the yields of the two enzymes.

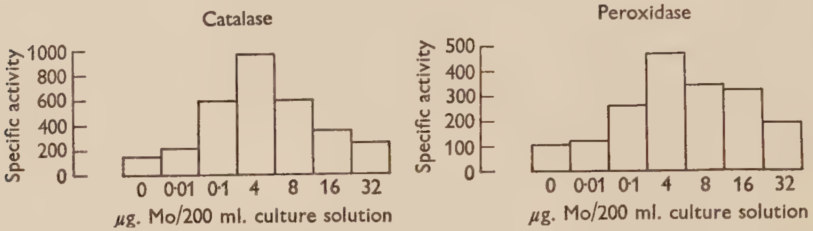

Fig. 2. Effect of different amounts of Mo in the culture solution on the yields of two iron-containing enzymes. Enzyme units as in Table 1.

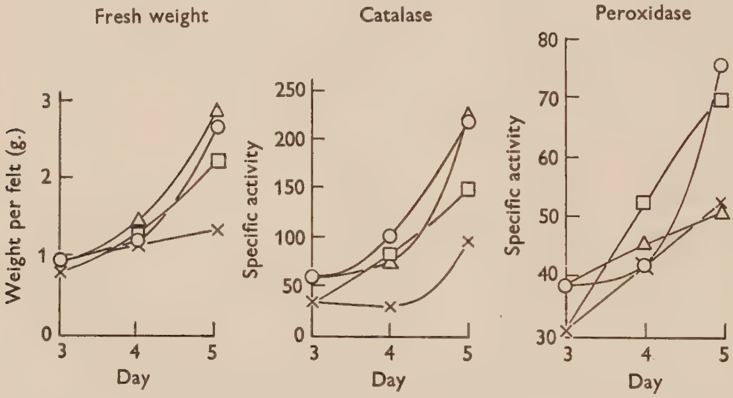

Fig. 3. Effect of concentration of Mo on fresh weight, and catalase and peroxidase contents of *Neurospora crassa* ○—○, normal felts; △—△, normal felts to which 4 μg. Mo/200 ml. culture solution was added aseptically after 3 days of growth. ×—×, Mo-deficient; □—□, Mo-deficient felts to which 4 μg. Mo/200 ml. culture solution was added as above. Enzyme units as in Table 1.

The effects on the weight and the production of the two iron-containing enzymes in the felts of adding Mo aseptically *in vivo* to normal cultures and to those deficient in Mo, after 3 days of growth, are illustrated in Fig. 3. The addition of Mo to the deficient felts resulted in a restoration of the weight to about 75 % of that of the normal felts after a further 2 days of growth. The amount of catalase recovered to 85 and 75 % of that in normal felts at the 4th and 5th day respectively. After addition of Mo to the deficient felts, the peroxidase activity at the 4th day exceeded the values in the control felts. On the 5th day, the peroxidase content was about 95 % of that in the normal felts.

It was not possible to reconstitute the two enzymes to the normal levels by adding 0·1 or 1 μg. Mo^{6+} (as sodium molybdate) or Mo^{5+} (as molybdenum pentachloride) to homogenates of felts deficient in Mo. Mixing extracts of normal mycelial felts with those from Mo-deficient felts proved that there was neither an inhibitor in the deficient felts nor an activator in the normal ones.

Effect of hydrogen peroxide

In order to establish whether Mo-deficiency was affecting the formation of the common substrate of the two iron-containing enzymes, hydrogen peroxide in graded amounts was added aseptically to the cultures of normal and deficient felts after 2 days of growth. Treated and non-treated felts were harvested immediately and others after a further 1, 2 and 3 days growth (viz. at the 3rd, 4th and 5th days of incubation). The results for the addition of 6 mg. H_2O_2/200 ml. culture medium is illustrated in Fig. 4. The addition of the H_2O_2 had no effect on the weight of felt of the control or the Mo-deficient cultures. On the 5th day catalase activity was partially restored (18 % of normal) and peroxidase activity showed a 50 % increase. There was no comparable effect in the control felts. The Mo content of the hydrogen peroxide added, determined by the bioassay method (Nicholas, 1952) was negligible. Thus no Mo was introduced in the hydrogen peroxide, as confirmed by finding no increase in the yield of Mo-deficient felts treated with this peroxide solution.

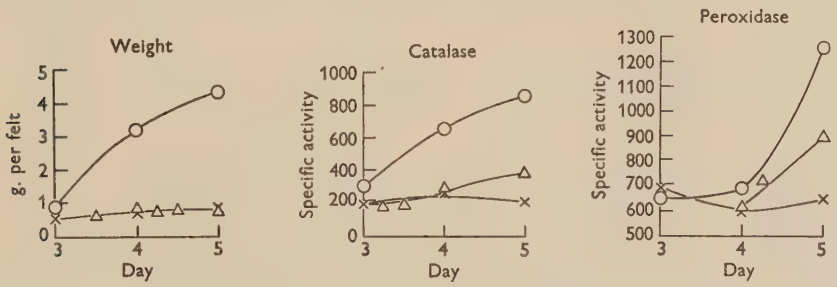

Fig. 4. Effect of hydrogen peroxide on fresh weight, catalase and peroxidase in *Neurospora crassa* grown with and without Mo. ○—○, normal felts; ×—×, Mo-deficient felts; △—△, Mo-deficient felts to which 6 mg. H_2O_2 was added *in vivo* to 200 ml. culture medium. Enzyme units as in Table 1.

Purification of catalase and peroxidase in relation to their molybdenum contents

To establish whether or not the effect of Mo-deficiency was on hydrogen peroxide production only, the two enzymes were purified and the Mo content and enzyme activity of the various fractions determined. To facilitate Mo determinations, 4 μc. ^{99}Mo/20 μg. Mo were incorporated/l. culture medium. Mycelial felts grown in 4 l. labelled media were used for each fractionation. The ^{99}Mo content of the various fractions were determined by radio-assay.

The results for catalase and peroxidase are given in Tables 1 and 2. The correlations between the value of ^{99}Mo/mg. protein and the specific activity of

Table 1. *Fractionation of catalase in* Neurospora crassa *wild type* 146

Molybdenum in medium labelled with 4 μC ^{99}Mo/20 μg. Mo/l. culture solution.

Fraction	Total protein (mg.)	Total enzyme units	Enzyme specific activity units/mg. protein	Recovery of enzyme (%)	Total ^{99}Mo (μmg.)	μmg. ^{99}Mo/mg. protein	Recovery of ^{99}Mo (%)
I Crude extract	702	126,000	179	100	1,180	1·68	100
II Ppt. from 0–50 % saturation with ammonium sulphate of fraction I. Fraction dissolved in 30 ml. 0·1 M-phosphate (pH 7·5)	94	6,450	68·5	5·1	138	1·47	11·7
III Ppt. from 50–100 % saturation with ammonium sulphate of fraction I. Fraction dissolved in 60 ml. 0·1 M-phosphate (pH 7·5)	350	82,920	237	66	245	0·71	20·5
IV Carbon slurry* added to fraction III (0·66 ml. carbon slurry/ml. enzyme). Supernatant fluid assayed	129	66,700	510	53	133	1·03	11·3
V Addition of equal vol. 95 % (v/v) ethanol to supernatant fluid from fraction III, at −15°. Ppt. dissolved in 25 ml. 0·1 M-phosphate (pH 7·5)	77	32,650	426	26	36	0·46	3·05
VI Ppt. from 0–70 % saturation with ammonium sulphate of fraction V. Ppt. dissolved in 10 ml. 0·1 M-phosphate (pH 7·5)	68	13,380	197	10·6	33	0·17	2·80
VII Ppt. from 70–100 % saturation with ammonium sulphate of fraction V. Ppt. dissolved in 10 ml. 0·1 M-phosphate (pH 7·5)	9·4	7,850	835	6·2	6·8	1·26	0·58
VIII Carbon treatment of fraction VII (0·2 ml. carbon slurry/ml. enzyme)	3·3	4,072	1,234	3·2	2·3	0·69	0·20

* G. 60 carbon slurry (100 mg. carbon/ml. H_2O) obtained from Darco Corporation, 42nd East Street, New York. Carbon slurry stirred slowly into the protein fractions at 0°, allowed to stand for 15 min. and then centrifuged at 3000 *g* for 15 min. at 0°.

Table 2. *Fractionation of peroxidase in* Neurospora crassa *wild type* 146

Molybdenum in medium labelled with 4 µC ^{99}Mo/20 µg. Mo/l. culture solution.

Fraction	Total protein (mg.)	Total enzyme units	Enzyme specific activity units/mg. protein	Recovery of enzyme (%)	Total ^{99}Mo (µmg.)	µmg. ^{99}Mo/mg. protein	Recovery of ^{99}Mo (%)
I Crude extract	885	1,295,360	1,463	100	3,060	3·46	100
II Addition of $\frac{1}{2}$ vol. of 95 % (v/v) ethanol in water to fraction I at −15°. Ppt. taken up in 50 ml. 0·1 M-phosphate (pH 7·5)	126	62,530	498	4·8	147	1·17	4·8
III Addition of another $\frac{1}{2}$ vol. of 95 % (v/v) ethanol in water to the supernatant fluid from fraction II at −15°. Ppt. taken up in 100 ml. 0·1 M-phosphate (pH 7·5)	354	851,016	2,404	66	214	0·65	7·0
IV Supernatant fluid from fraction III	420	456,220	1,091	35	2,405	5·73	79
V Carbon added to fraction III (0·66 ml. carbon slurry*/ml. enzyme)	100	390,000	3,900	30	110	1·13	3·6
VI Ppt. from 0–50 % saturation with ammonium sulphate of fraction V, dissolved in 50 ml. 0·1 M-phosphate (pH 7·5)	60	84,600	1,410	6·5	29	0·48	0·95
VII Ppt. from 50–100 % saturation with ammonium sulphate of fraction V dissolved in 20 ml. 0·1 M-phosphate (pH 7·5)	11	178,420	16,220	14	5	0·46	0·16
VIII Supernatant fluid IV left overnight at 0° then centrifuged. Ppt. dissolved in 100 ml. 0·1 M-phosphate (pH 7·5)	226	438,440	1,940	34	96	0·43	3·1
IX Carbon added to fraction VIII (0·66 ml. carbon slurry*/ml. enzyme)	17	37,160	2,187	2·9	31	1·87	1·0
X Ppt. from 0–50 % saturation with ammonium sulphate of fraction IX, dissolved in 20 ml. 0·1 M-phosphate (pH 7·5)	5·9	28,200	4,780	2·2	2·8	0·47	0·09
XI Ppt. from 50–100 % saturation with ammonium sulphate of fraction IX	0·96	15,620	16,270	1·2	0·4	0·42	0·013

* G. 60 carbon slurry (100 mg. carbon/ml. H_2O) obtained from Darco Corporation, 42nd East Street, New York. Carbon slurry stirred slowly into the protein fractions at 0°, allowed to stand for 15 min. and then centrifuged at 3000 *g* for 15 min. at 0°.

catalase or peroxidase (enzyme units/mg. protein) are illustrated in Figs. 5 and 6. It is clear that there was no positive correlation between the Mo content of either enzyme and its activity, so that molybdenum does not appear to concentrate in these enzymes as they are purified. Further, the addition of Mo^{6+} as sodium molybdate or Mo^{5+} as molybdenum pentachloride did not stimulate the activity of the purified fractions of either enzyme. Thus molybdenum seems unlikely to be a constituent of these two iron-containing enzymes.

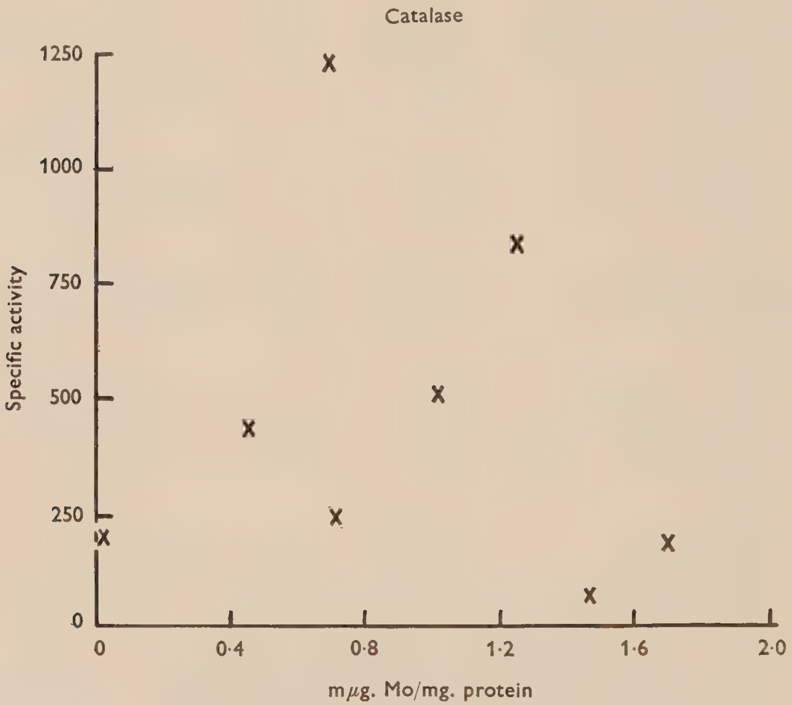

Fig. 5. Relation between specific activity of catalase (units/mg. protein) and mμg. ^{99}Mo/mg. protein in the various protein fractions of the fungus.

DISCUSSION

Although a deficiency of molybdenum depressed catalase and peroxidase activities in *Neurospora crassa*, possibly by decreasing the amount of their common substrate hydrogen peroxide, the mechanism whereby this occurs is not clear. It is known that riboflavin and flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) react with oxygen to form hydrogen peroxide (Theorell, 1951). It has also been shown that some flavoprotein enzymes may be decreased in activity when molybdenum is deficient. Thus nitrate reductase in fungi, bacteria and green plants, which has FAD as the native flavin coenzyme, has been shown to contain molybdenum which undergoes a valence change involving Mo^{6+} and Mo^{5+} during the enzymic reduction of nitrate (Nicholas & Stevens, 1955). In *Pseudomonas fluorescens* grown at low oxygen pressure, the electron transfer system is mainly iron-dependent,

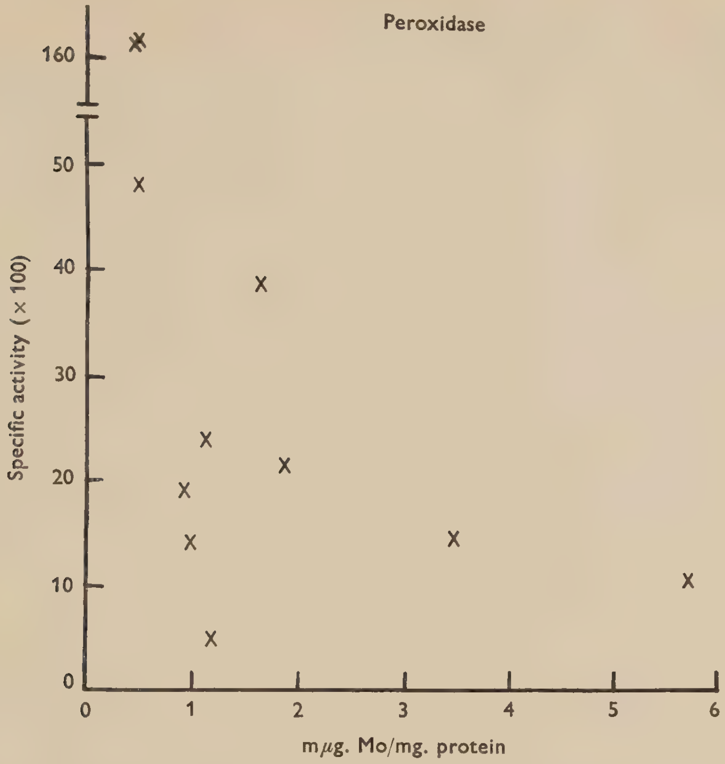

Fig. 6. Relation beteeen specific activity of peroxidase (units/mg. protein × 100 and mμg. ^{99}Mo/mg. protein, in the various protein fractions of the fungus.

whereas under high oxygen pressures a molybdo-flavoprotein system became dominant (Lenhoff, Nicholas & Kaplan, 1956). Thus a deficiency in hydrogen peroxide in *Neurospora crassa* may well arise from a depletion in molybdenum-dependent flavoproteins which have the capacity to react with oxygen to form peroxides, this in turn diminishing the degree of induced formation of catalase and peroxidase.

Thanks are due to Mr C. P. Lloyd-Jones, B.Sc., for providing a counting service for the radioassay of ^{99}Mo and to Mr G. Mabey for technical assistance. Mr G. H. Jones kindly made the photographic records.

REFERENCES

Feinstein, R. N. (1949). Perborate as substrate in a new assay of catalase. *J. biol. Chem.* **180**, 1197.

Lenhoff, H. M., Nicholas, D. J. D. & Kaplan, N. O. (1956). Effects of oxygen, iron, and molybdenum on routes of electron transfer in *Pseudomonas fluorescens*. *J. biol. Chem.* **220**, 983.

Lowry, O. H., Rosebrough, N. L., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

Nicholas, D. J. D. & Fielding, A. H. (1951). Use of *Aspergillus niger* (M) for the determination of magnesium, zinc, copper and molybdenum available in soils to crop plants. *J. hort. Sci.* **26**, 125.

Nicholas, D. J. D. (1952). The use of fungi for determining trace metals in biological materials. *Analyst*, **77**, 629.

Nicholas, D. J. D., Nason, A. & McElroy, W. D. (1954). Molybdenum and nitrate reductase. I. Effect of molybdenum on the *Neurospora* enzyme. *J. biol. Chem.* **207**, 341.

Nicholas, D. J. D. & Nason, A. (1954*a*). Molybdenum and nitrate reductase. II. Molybdenum as a constituent of nitrate reductase. *J. biol. Chem.* **207**, 353.

Nicholas, D. J. D. & Nason, A. (1954*b*). Mechanism of action of nitrate reductase from *Neurospora*. *J. biol. Chem.* **211**, 183.

Nicholas, D. J. D. & Nason, A. (1954*c*). Diphosphopyridine-nucleotide-nitrate reductase from *Escherichia coli*. *J. Bact.* **69**, 580.

Nicholas, D. J. D. & Nason, A. (1955). Role of molybdenum as a constituent of nitrate reductase from soybean leaves. *Plant Physiol.* **30**, 135.

Nicholas, D. J. D. & Stevens, H. M. (1955). Valency changes of molybdenum during the enzymatic reduction of nitrate in *Neurospora*. *Nature, Lond.* **176**, 1066.

Nicholas, D. J. D. (1956). Effect of molybdenum deficiency on catalase and peroxidase in *Neurospora*. *Nature, Lond.* **178**, 148.

Smith, F. G., Robinson, W. B. & Stotz, E. (1949). A colorimetric method for the determination of peroxidase in plant material. *J. biol. Chem.* **179**, 881.

Theorell, H. (1951). In *The Enzymes*. Ed. Sumner, J. B. & Myrbäck, K. **2**, i, p. 335. New York: Academic Press.

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NICHOLAS, D. J. D. & COMMISSIONG, K. (1957). *J. gen. Microbiol.* **17**, 699–707

Effects of Molybdenum, Copper and Iron on Some Enzymes in *Neurospora crassa*

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SUMMARY: Diverse enzyme changes were obtained in *Neurospora crassa* grown at deficient, optimal and toxic levels of Mo, Cu and Fe. These included mutual antagonisms between the metals, single effects of one element or additive ones of more than one element. Cytochrome *c* oxidase content, enhanced by Cu treatment, was decreased by increasing Mo; the reverse relationship was obtained with nitrate reductase. Cytochrome *c* and nitrite reductases, however, were depressed by amounts both of Cu and Mo which were toxic to growth; the effects were additive. Single effects were as follows: TPNH diaphorase and acid phosphatase were depressed by Cu and Mo excess respectively; the iron enzymes and the two diaphorases were more active when Fe was increased from deficiency to sufficiency level. High iron concentrations increased nitrate reductase but depressed the nitrite system. Molybdenum is shown to inhibit acid phosphatase competitively *in vitro*, presumably by forming a molybdo-phosphate addition compound with the substrate. Cu dispersed this complex and restored the enzyme activity to normal. Vanadate or tungstate had a similar effect to molybdate in depressing the enzyme but Cu was found to reverse the inhibition.

Thus it appears that antagonisms between metals in nutrition experiments may be related to their effects on certain enzymes as illustrated here for Cu, Mo and Fe in *Neurospora*.

Antagonisms between nutrients are well established in plants and animals but little is known how these affect cell metabolism. Healy, Cheng & McElroy (1955) showed that the effect of Co excess in depressing iron-containing enzymes in *Neurospora crassa* was partially reversed by increasing Fe in the culture solution. van Reen (1953) demonstrated that Zn toxicity in the rat decreased the amount of depressed liver catalase and cytochrome *c* oxidase and that this effect was remedied by adding copper sulphate to the diet.

This paper amplifies a preliminary report in which it was shown that Cu and Mo were reciprocally antagonistic towards cytochrome *c* oxidase and nitrate reductase in *Neurospora crassa* (Commissiong & Nicholas, 1957); the effects of iron concentration on some enzymes are also considered.

METHODS

Culture medium. Neurospora crassa, macroconidial wild type 5297a, was grown in the following medium: sucrose, 20 g.; Na tartrate, 1 g.; KH_2PO_4, 3 g.; $MgSO_4.7H_2O$, 0·5 g.; NaCl, 0·1 g.; $CaCl_2$, 0·1 g. After removing Fe, Cu and Mo from the macronutrients (Nicholas, 1952), the following micronutrients were added: biotin, 5 μg.; Zn as $ZnSO_4.7H_2O$, 200 μg.; Mn as $MnCl_2.4H_2O$, 20 μg.; Cu as $CuSO_4.5H_2O$ at 0, 0·08 mg. or 10 mg.; Mo as

$Na_2MoO_4.2H_2O$ at 0, 0·02 mg. or 200 mg.; Fe as $FeCl_3.6H_2O$ at 0, 0·2 mg. or 35 mg.; distilled water, 1 l.; pH 4·8. The three concentrations of Fe, Cu and Mo represented deficiency (0), normal (1), and toxicity concentrations (2), based on the dry weight yields of the felts.

Purification of culture media. Fe, Cu and Mo were removed from the macronutrients by the copper co-precipitation and quinoline methods (Nicholas, 1952). The micronutrient elements Fe, Cu, Mn, Zn and Mo were used as 'spectroscopically pure' compounds supplied by Johnson and Mathey (Hatton Garden, London, E.C. 1). Biotin (25 μg.) dispensed in phosphate buffer (pH 7) was supplied in ampoules by the Ashe Laboratories (Leatherhead, Surrey). The culture medium (200 ml.) was dispensed in 1 l. Erlenmeyer flasks, and after sterilizing at 10 lb./sq.in. for 10 min. and cooling, various amounts of Fe, Cu and Mo were added to their respective containers. The media were then inoculated with a spore suspension of *Neurospora crassa* 5297a in glass-distilled water and incubated for 4 days at 30° in the dark. The cultures were shaken periodically to prevent sporulation.

Preparation of cell-free extracts. The mycelial felts from each of the twenty-seven treatments, collected separately in sintered Buchner funnels, were washed thoroughly with glass-distilled water and frozen overnight at −17°. They were then homogenized, in groups of nine, in four times their weight of cold 0·1 M-phosphate buffer (pH 7·5) in a mortar and then in a Ten Broeck glass macerator at 0°. The homogenates were centrifuged at 3000 *g* for 10 min. at 4° and the supernatant solutions used for enzyme assays.

Cofactors and other compounds. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) (both of 95 % purity) and cytochrome *c* were obtained from the Sigma Chemical Company, U.S.A. Reduced DPN was prepared enzymically by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1952) and reduced TPN by using the isocitric dehydrogenase enzyme from acetone powder of pig heart. Boiled pig heart extract, centrifuged at 18,000 *g* for 20 min. at 4° was used as a source of flavine adenine dinucleotide (FAD).

Catalase activity was measured at 37° by the perborate method of Feinstein (1949). The reaction mixture contained 8 ml. 1·5 % (w/v) $NaBO_3.4H_2O$ (pH 6·8); 1·9 ml. 0·1 M-phosphate buffer (pH 6·8); 0·1 ml. enzyme extract; this was incubated at 37° for 5 min. Then 10 ml. 2 N-H_2SO_4 was added and the solution titrated with 0·05 N-$KMnO_4$. The values are expressed as m-mole perborate degraded/5 min./mg. protein.

Peroxidase was determined by the spectrophotometric method of Smith, Robinson & Stotz (1949). The reaction mixture contained 1·45 ml. McIlvaine's buffer (pH 6·0); 1·0 ml. 10^{-3} reduced M-2:6-dichloroindophenol; 0·5 ml. 0·1 M-H_2O_2; 0·05 ml. enzyme extract. The oxidation of the dye was followed at 625 mμ. at 15 sec. intervals. The unit of activity was the change in log I_0/I of 0·001/min. calculated between 15 and 75 sec./mg. protein.

DPNH and TPNH *oxidases* ('diaphorase' systems) were measured by following dye reduction at 625 mμ. The reaction mixture used was: 2·85 ml. 0·1 M-phosphate buffer (pH 7·5); 0·15 ml. 1×10^{-3} M-2:6-dichloroindophenol;

0·1 ml. 0·1 M-KCN; 0·05 ml. DPNH (10^{-3} M) or TPNH (2 μmole/ml.); 0·05 ml. enzyme extract.

Cytochrome c *oxidase* was estimated by the method of Smith & Stotz (1949). The reaction mixture contained 1·95 ml. 0·1 M-phosphate (pH 6·8); 1·0 ml. 10^{-3} reduced M-2:6-dichloroindophenol; 0·2 ml. 1×10^{-4} M-cytochrome *c*; 0·05 ml. enzyme extract. The unit of activity was the change in log I_0/I of 0·001/min. calculated between 15 and 75 sec./mg. protein.

DPN-*cytochrome* c *reductase* was measured at 551 mμ. The reaction solution contained 2·65 ml. 0·1 M-phosphate buffer (pH 7·5); 0·03 ml. 0·1 M-KCN; 0·2 ml. 0·1 M-$MgCl_2.6H_2O$; 0·1 ml. cytochrome *c*, 3×10^{-3} M; 0·1 ml. DPNH (10^{-3} M); 0·1 ml. enzyme extract.

Nitrate reductase was measured by the method of Nicholas, Nason & McElroy (1954). The assay method consisted of adding 0·1 ml. enzyme extract to a solution containing 0·1 ml. 0·1 M-KNO_3; 0·1 ml. boiled pig heart extract; 0·1 ml. 10^{-4} M-KCN; 0·1 ml. 1×10^{-3} M-TPNH; 0·1 ml. 0·1 M-phosphate buffer (pH 7·5). The mixture was incubated for 10 min. at 25° and the reaction stopped by adding 1·0 ml. 0·001 % (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride reagent followed by 0·5 ml. 1 % (w/v) sulphanilamide reagent. The volume was made up to 5 ml. with glass-distilled water and read on the Spekker absorptiometer, after 10 min., at 540 mμ. Control tests without TPNH were used to correct for the turbidity of the enzyme preparation. Units of activity are expressed as mμmole NO_2^- formed in 10 min./mg. protein.

Nitrite reductase was determined by the method described by Medina & Nicholas (1957). The test consisted of adding 0·1 ml. enzyme to a solution containing 0·2 ml. 10^{-3} M-$NaNO_2$; 0·1 ml. boiled pig heart extract; 0·1 ml. DPNH (10^{-3} M) and 0·25 ml. 0·1 M-pyrophosphate buffer (pH 7·5). After 10 min. incubation at 25°, 0·1 ml. 1·0 M-Ba acetate and 3·15 ml. cold absolute ethanol were added. The mixture was stirred well, left at 4° for 30 min. and the precipitate centrifuged down; 0·5 ml. of the supernatant fluid was then taken and the colour developed as in the nitrate reductase assay.

Alkaline and acid phosphatases were estimated by the method of Bessey, Lowry & Brock (1946) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol formed in 30 min./mg. protein was determined at alkaline and acid pH.

Protein was determined by the Folin method as described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Enzyme assays were made on the extracts from the mycelial felts of each of the twenty-seven treatment combinations; the results were analysed statistically.

Iron-containing enzymes

Effect of iron concentration. Catalase, peroxidase, cytochrome *c* oxidase and its reductase were all affected by an increase in iron concentration as illustrated in Fig. 1. Their means showed in each case a significant increase from the Fe_0

to the Fe_1 level. Cytochrome *c* oxidase was depressed at Fe concentrations that depressed growth.

Effect of copper. Of the four iron-containing enzymes examined, only cytochrome *c* oxidase and cytochrome *c* reductase were significantly affected by the copper treatment. From deficiency to the toxicity level of the metal, based on growth measurements, catalase activity decreased steadily.

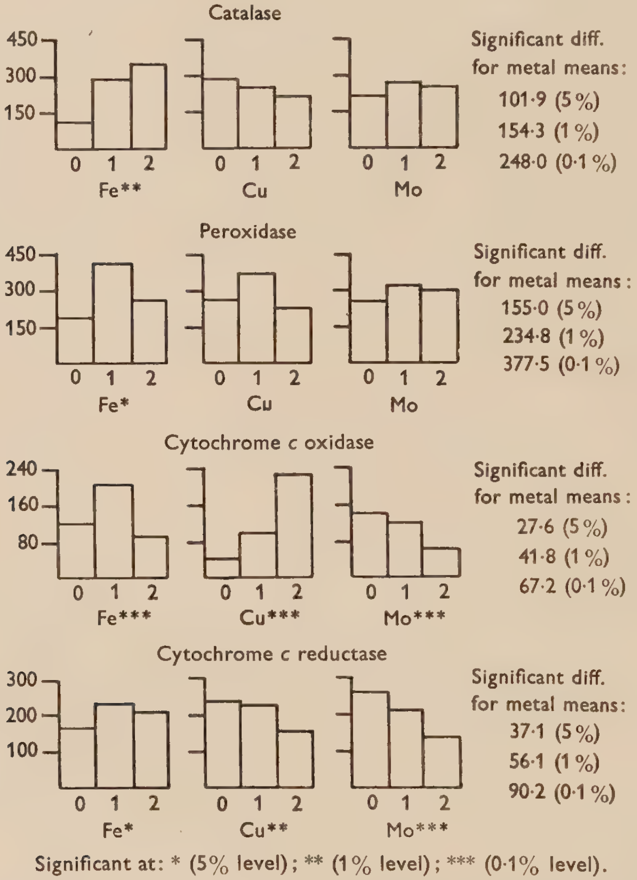


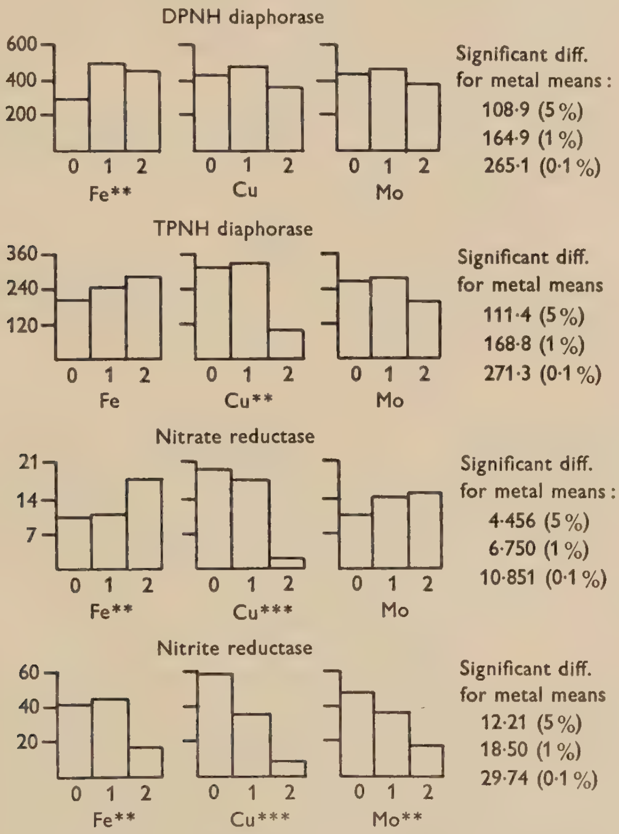
Fig. 1. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

The response of cytochrome *c* oxidase and cytochrome *c* reductase to an increase in copper varied markedly. Cytochrome *c* oxidase was significantly increased from the deficiency to the high levels that depressed yields of the felts by 50%. Cytochrome *c* reductase, however, was significantly depressed at the highest concentration of the metal.

Effect of molybdenum. The enzymes differed in their response to molybdenum. Although the mean values for catalase or peroxidase activity were not significantly different for the various Mo treatments there was a tendency for an increase in activity of these two enzymes from deficiency to the sufficiency level, followed by a depression at concentrations that depressed felt weight.

Cytochrome *c* reductase was significantly depressed by increasing Mo from deficiency to values that were associated with a depression in the yields of felts. The oxidase showed a similar trend and the difference between the Mo_1 and Mo_2 treatments was significant.

In vitro *experiments.* The only effect of adding Cu or Mo *in vitro* at 1 µg. to 0·1 ml. extract of normal felts was to increase the endogenous rates of peroxidase and cytochrome *c* oxidase.



Significant at: * (5% level); ** (1% level); *** (0·1% level).

Fig. 2. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

Flavoprotein enzymes

Effect of iron. The contents of DPNH diaphorase, nitrate and nitrite reductases were significantly changed by the iron treatments as shown in Fig. 2. The TPNH diaphorase showed a gradual increase from the deficiency to the toxicity level, based on yield of felts. The DPNH diaphorase was significantly increased in activity from the Fe_0 to the Fe_1 level.

Nitrate and nitrite reductases showed opposite effects in their response to iron at the highest concentration; the former enzyme activity was stimulated whereas that for the nitrite reductase was depressed.

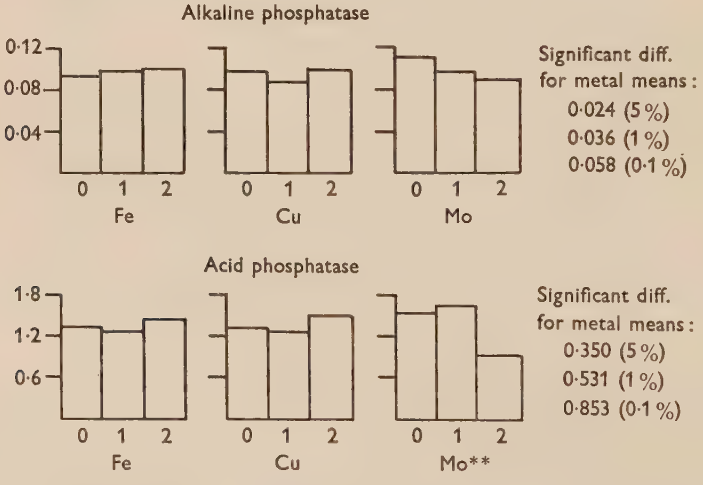
Effect of copper. Increasing copper from sufficiency to toxicity concentrations based on the weight of mycelial felts, depressed both DPNH and TPNH diaphorases, these effects, however, just failed to reach significance for the latter enzyme. Nitrate and nitrite reductases were significantly depressed at the highest concentrations of copper.

Effect of molybdenum. Molybdenum status did not significantly affect the diaphorase enzymes although a high level of the micronutrient tended to depress them. Nitrate reductase increased from $Mo_0 \rightarrow Mo_1 \rightarrow Mo_2$ whereas nitrite reductase showed the opposite effect.

In vitro *studies.* The addition of Cu or Mo at 1 μg. to 0·1 ml. extract of normal felts had no effect either on DPNH or TPNH diaphorases. Nitrate and nitrite reductases were, however, depressed at this Cu concentration.

Phosphatases

Alkaline phosphatase was not changed by the treatments given whereas the acid phosphatase was significantly depressed when molybdenum was in excess as shown in Fig. 3.



Significant at: * (5% level); ** (1% level); *** (0·1% level).

Fig. 3. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

In vitro *experiments.* Alkaline phosphatase was unchanged by the addition either of Cu or Mo to the extracts. The results of *in vitro* experiments with acid phosphatase are shown in Table 1. Molybdate markedly depressed acid phosphatase whereas Cu stimulated its activity. The addition of Cu partially reversed the inhibition by molybdate whereas Fe, Mn and Zn were without effect. Vanadate or tungstate also markedly depressed the enzyme.

Table 1. *The effect of metal addition* in vitro *on the activity of acid phosphatase*

Values as percentage of normal

Control	$FeCl_3$	$MnCl_2$	$ZnSO_4$	$CuSO_4$	$(NH_4)_6Mo_7O_{24}.4H_2O$	NH_4VO_3	$Na_2WO_4.2H_2O$	Cu+Mo
100	94	100	107	118	30	19	20	46

1 μg. of each *metal* added to the reaction mixture which consisted of: 1 ml. acid phosphatase reagent and 0·05 ml. enzyme extract incubated at 38° for 30 min. and made to a final volume of 5 ml. with 0·06 N-NaOH.

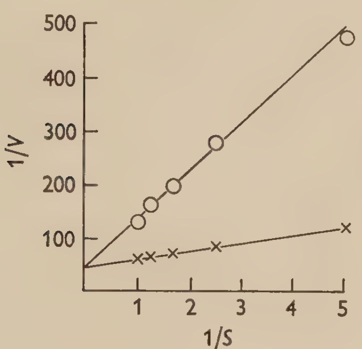


Fig. 4

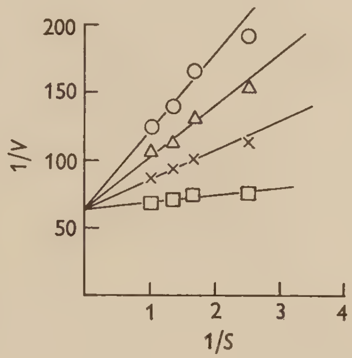


Fig. 5

Fig. 4. Lineweaver-Burk (1934) plot of the competitive inhibition of acid phosphatase activity by sodium molybdate. Assay mixtures consisted of 1 ml. acid phosphatase reagent, 0·05 ml. enzyme extract and 0·05 ml. of either distilled water or a solution of Na_2MoO_4 of 8 μg. Mo/ml. The mixtures were incubated at 38° for 30 min. and then 3·9 ml. of 0·06 N-NaOH were added to make a final volume of 5 ml. The intensity of the *p*-nitrophenol colour developed was read on the Spekker Absorptiometer at 540 mμ. 1/V, reciprocal of the intensity of the *p*-nitrophenol at 540 mμ. × 10^4; 1/S, reciprocal of substrate concentration where the normal substrate concentration is considered as unity. ×—×, no inhibitor; ○—○, with molybdate.

Fig. 5. Lineweaver-Burk (1934) plot showing competitive inhibition of copper by molybdate on acid phosphatase (*in vitro*). Assay mixtures consisted of 1 ml. acid phosphatase reagent, 0·05 ml. enzyme extract, 0·05 ml. $Na_2MoO_4.2H_2O$ at 0, 1·0, 2·1 or $5·2 \times 10^{-6}$ M-$Na_2MoO_4.2H_2O$ and 0·05 ml. $CuSO_4.5H_2O$ at 0·6, 0·9, 1·2 and $1·5 \times 10^{-5}$ M. The mixtures were incubated at 38° for 30 min. and then 3·85 ml. 0·06 N-NaOH were added to make a final volume of 5 ml. The intensity of the *p*-nitrophenol colour developed was read on the Spekker absorptiometer at 540 mμ. 1/V, reciprocal of the intensity of the *p*-nitrophenol at 540 mμ. × 10^4; 1/S, reciprocal of the amount of copper added per assay. □—□, no molybdenum; ×—×, $1·0 \times 10^{-6}$ M-$Na_2MoO_4.2H_2O$; △—△, $2·1 \times 10^{-6}$ M-$Na_2MoO_4.2H_2O$; ○—○, $5·2 \times 10^{-6}$ M-$Na_2MoO_4.2H_2O$.

Fig. 4 shows that molybdate inhibits the enzyme competitively. The K_m for the enzyme-substrate complex was calculated to be $1·6 \times 10^{-3}$ M and the dissociation constant for the enzyme-inhibitor complex was $0·68 \times 10^{-5}$ M.

The results in Fig. 5 demonstrate the competitive nature of the inhibition of Cu by molybdate (*in vitro*) on acid phosphatase.

DISCUSSION

Although it is well established that Mo and Cu are antagonistic in animal nutrition experiments, little work has been done on enzyme changes which occur in the tissues under these conditions. Changes in enzyme pattern in *Neurospora crassa* given different amounts of Cu, Mo and Fe, reported in this paper, illustrate that some key enzymes, drastically decreased by one of these metals, may be partially restored by increasing one of the other two in the culture solution. Thus the activity of cytochrome *c* oxidase was enhanced by adding Cu, whereas amounts of Mo that decreased felt weight, decreased it. A similar result was obtained by van Reen (1953) in the rat where adding Cu alleviated the effect of Zn in depressing cytochrome *c* oxidase in the liver. The effect of Cu in increasing this enzyme is in keeping with the suggestion that Cu is a component of the DPNH oxidase system (Green, Basford & Mackler, 1956).

The opposite effect was obtained with nitrate reductase, a molybdo-flavo-protein, where an increase in Mo enhanced its activity and Cu depressed it. The addition of Cu also depressed the enzyme *in vitro* but the addition of Mo failed to restore the low activity of nitrate reductase prepared from Mo-deficient felts (Nicholas, Nason & McElroy, 1954).

Cytochrome *c* reductase was decreased when Cu was in excess, in contrast to a substantial increase in its oxidase, whereas iron treatments increased both enzymes. The effects of Cu excess on cytochrome *c* reductase is unlikely to be caused by interference with haem-iron because the oxidase activity is enhanced under similar conditions. Cu excess may, therefore, affect the non-haem-iron which is only present in the reductase system.

A number of enzymes decreased by toxic concentrations of one of the metals were either not increased by varying the concentrations of the other two in the culture solution or were depressed further. Thus, it appears that nutrient antagonisms are reflected by some enzymes only and in this study these were metallo-proteins.

Acid phosphatase was depressed by increasing Mo from sufficiency to toxicity levels (based on dry weight of the felts), thus confirming a similar effect observed in higher plants (Spencer, 1954). Molybdate is shown to be a competitive inhibitor of copper in acid phosphatase *in vitro*. Vanadate and tungstate had a similar inhibitory effect to molybdate, presumably because they too form complexes in acid solution with *p*-nitrophenyl phosphate, the substrate for the phosphatase. It is noteworthy that alkaline phosphatase was unaffected because the substrate-molybdate complex is unstable at pH 10·3; the pH at which the assay is made.

In these experiments, substantial differences were required between mean values before significant effects were obtained. This was in the main due to the limited number of replicates that could be conveniently handled in enzyme work of this nature. For this reason some effects known to occur in previous experiments just failed to reach significance in these studies, e.g. a greater catalase and peroxidase activity in felts given optimal Mo (Nicholas, 1956).

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REFERENCES

Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum. *J. biol. Chem.* **164**, 321.

Commissiong, K. & Nicholas, D. J. D. (1957). Effects of molybdenum and copper on some enzymes in *Neurospora*. *Nature, Lond.* **180**, 555.

Feinstein, R. N. (1949). Perborate as substrate in a new assay of catalase. *J. biol. Chem.* **180**, 1197.

Green, D. E., Basford, R. E. & Mackler, B. (1956). The role of iron and copper in terminal electron transfer in *Inorganic Nitrogen Metabolism*, Ed. McElroy, W. D. & Glass, B. p. 628. Baltimore: The Johns Hopkins Press.

Healy, W. B., Cheng, S. & McElroy, W. D. (1955). Metal toxicity and iron deficiency effects on enzymes in *Neurospora*. *Arch. Biochem. Biophys.* **54**, 206.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265.

Medina, A. & Nicholas, D. J. D. (1957). Interference by reduced pyridine nucleotides in the diazotization of nitrite. *Biochim. biophys. Acta*, **23**, 440.

Nicholas, D. J. D. (1952). The use of fungi for determining trace metals in biological materials. *Analyst*, **77**, 629.

Nicholas, D. J. D. (1956). Effect of molybdenum deficiency on catalase and peroxidase in *Neurospora*. *Nature, Lond.* **178**, 148.

Nicholas, D. J. D., Nason, A. & McElroy, W. D. (1954). Molybdenum and nitrate reductase. 1. Effect of molybdenum deficiency on the *Neurospora* enzyme. *J. biol. Chem.* **207**, 341.

Pullman, M. E., Colowick, S. P. & Kaplan, N. O. (1952). Comparison of diphosphopyridine nucleotide with its deaminated derivative in various enzyme systems. *J. biol. Chem.* **194**, 593.

Reen, R. van (1953). Effects of excessive dietary zinc in the rat and the interrelationship with copper. *Arch. Biochem. Biophys.* **46**, 337.

Smith, F. G., Robinson, W. B. & Stotz, E. (1949). A colorimetric method for the determination of peroxidase in plant material. *J. biol. Chem.* **179**, 881.

Smith, F. G. & Stotz, E. (1949). A colorimetric method for the determination of cytochrome oxidase. *J. biol. Chem.* **179**, 891.

Spencer, D. (1954). The effect of molybdate on the activity of tomato acid phosphatases. *Aust. J. biol. Sci.* **7**, 151.

(*Received* 17 *June* 1957)

Newton, B. A. (1957). *J. gen. Microbiol.* **17**, 708–717

Nutritional Requirements and Biosynthetic Capabilities of the Parasitic Flagellate *Strigomonas oncopelti*

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SUMMARY: A study of the nutritional requirements of the trypanosomid flagellate *Strigomonas* (*Herpetomonas*) *oncopelti* has led to the development of a chemically defined growth medium containing methionine, thiamine, nicotinamide, *p*-aminobenzoic acid, glucose, salts and trace metals. Fractionation and analysis of organisms grown in this defined medium supplemented with trace amounts of various ^{14}C-labelled substrates has thrown light upon the ability of growing organisms to utilize carbon derived from glucose, acetate, a number of amino acids, purines and pyrimidines for the synthesis of cellular components.

Metabolic studies of the Trypanosomidae have in general been confined to studies of catabolism and there is little known about the pathways of biosynthesis in these organisms. The development of a simple chemically defined growth medium for the parasitic flagellate *Strigomonas* (*Herpetomonas*) *oncopelti* (Newton, 1956*a*) permits a detailed study of the biosynthetic capabilities of an actively growing trypanosomid.

Strigomonas oncopelti was first isolated in bacteria-free culture by Noguchi & Tilden in 1926; it is parasitic in the digestive tract of hemipterous insects and in latex plants. Lwoff (1937) reported that *S. oncopelti* can be cultivated in certain peptones or in alkaline hydrolysates of silk when these are supplemented with thiamine; he also observed that this organism, in contrast to other members of the Trypanosomidae, does not require haematin for growth in peptone media. This latter finding has recently been confirmed by Nathan, Cowperthwaite, Carsted & Hutner (1955), but these workers found, unexpectedly, that their strain of *S. oncopelti* did require haematin for growth in a chemically defined medium containing seventeen amino acids, a complete vitamin supplement and a selection of nucleic acid derivatives. The strain of *S. oncopelti* used for the work described in the present paper did not require haematin in either peptone or the defined media. Studies of the catabolic metabolism of *S. oncopelti* were recently described by Clausen (1955) and Ryley (1955) and the latter author pointed out that there are striking similarities between this organism and *Trypanosoma lewisi*, especially with regard to their carbohydrate metabolism. The present paper describes the nutritional requirements of *S. oncopelti* and the ability of actively growing organisms to utilize various ^{14}C-labelled substrates for the synthesis of cellular components; some of these results have been reported in a preliminary communication (Newton, 1956*b*).

METHODS

Organism. A bacteria-free culture of *Strigomonas* (*Herpetomonas*) *oncopelti* was kindly supplied by Dr Muriel Robertson, F.R.S. (Lister Institute of Preventive Medicine, London).

Conditions of cultivation. Stock cultures were maintained in 5 ml. volumes of 3 % (w/v) Evans bacteriological peptone containing 0·5 % (w/v) sodium chloride and 0·5 % (w/v) glucose; initial pH 7·4. The medium was sterilized by autoclaving at 15 lb./sq.in. for 20 min. Cultures were incubated at 25° and subcultured every other day.

Growth tests. The nutritional requirements of *Strigomonas oncopelti* were determined in defined media according to the general method of Gladstone (1937). A 'complete' defined medium was prepared containing glucose, salts and trace metals in the amounts shown in Table 1 and in addition 18 amino acids (glycine, L-aspartic and L-glutamic acids, L-arginine, DL-methionine, L-lysine, DL-threonine, L-proline, DL-tryptophan, L-tyrosine, L-cysteine, DL-serine, DL-phenylalanine, L-histidine, L-valine, DL-alanine, L-leucine and DL-isoleucine) at a final concentration of 200 μg. L-isomer/ml.; purines and pyrimidines (adenine, xanthine, hypoxanthine, guanine, thymine, cytosine, uracil) at a final concentration of 20 μg./ml. and growth factors (thiamine, pyridoxin, riboflavin, calcium pantothenate, nicotinic acid, biotin, *p*-aminobenzoic acid) at a final concentration of 1 μg./ml. except for biotin which was used at 0·001 μg./ml. Further batches of medium were prepared to test the effect of omission of the above components either singly or in groups. Organisms to be used as inocula in growth tests were grown in the complete defined medium for 24 hr. at 25° and were washed three times in the medium to be tested; a standard inoculum of *c.* 2 × 10^5 organisms/ml. medium was used in all cases and the amount of growth was measured by counting the organisms at 24 hr. intervals during incubation at 25°.

Table 1. *Minimal defined growth medium for* Strigomonas oncopelti

	mg.		g.
Methionine	200·0	Glucose	5·0
Thiamine	1·0	NH_4Cl	5·0
Nicotinamide	1·0	$Na_2HPO_4.2H_2O$	0·25
p-Aminobenzoic acid	1·0	KH_2PO_4	0·08
		NaCl	9·0
		KCl	0·42

Trace metal solution*, 10 ml.
Glass-distilled water to 1 l. Initial pH value 7·2.
Medium sterilized by autoclaving at 10 lb./sq.in. for 10 min.

* Trace metal solution: $MgSO_4.7H_2O$, 20 g.; $FeSO_4$, 0·5 g.; $ZnSO_4.7H_2O$, 0·5 g.; $MnSO_4.3H_2O$, 0·5 g.; $CuSO_4.5H_2O$, 0·5 g. made up to 1 l. in 0·1N-H_2SO_4.

Counting the organisms. Samples (1 ml.) of cultures were fixed with 0·05 ml. 40 % (w/v) formaldehyde in water. Four counts were made on each sample in a Neubauer haemocytometer, a minimum of 300 organisms being counted in each case.

Conditions of growth for incorporation experiments. Five ml. of a 48 hr. culture in the minimal defined medium (Table 1), or in modifications of this medium to be described below, were used as inoculum for 50 ml. lots of the same medium contained in 150 ml. conical flasks, to which had been added trace amounts of ^{14}C-labelled substrates; the initial count of organisms was *c.* 15×10^5/ml. in all experiments. An experimental growth period of 24 hr. was used, resulting in a 2- to 3-fold increase in number of organisms.

Harvesting and fractionation of organisms. The organisms were harvested by centrifugation and washed three times with a saline solution (0·9 %, w/v, NaCl + 0·42 %, w/v, KCl) before fractionation. The fractionation procedure adopted was essentially the same as that described by McQuillen & Roberts (1954): 5 % (w/v) trichloroacetic acid (TCA) for 1 hr. at 5° extracts acid-soluble constituents (fraction 1); 75 % (v/v) ethanol in water for 30 min. at 50° extracts ethanol-soluble protein and some lipid; further extraction with ether (1 vol.) + 75 % (v/v) ethanol in water (1 vol.) for 15 min. at 37° removes further fatty material and protein. The ethanol and ether + ethanol extracts are combined, addition of equal volumes of ether and water then results in division of two phases: ethanol-soluble protein (fraction 2) remains in the aqueous phase and the lipid (fraction 3) enters the ether phase; finally, 5 % (w/v) TCA for 15 min. at 100° extracts nucleic acids (fraction 4). The residue after ether washing represents the main protein fraction of the organisms (fraction 5) which was dissolved in N-NH_4OH before determinations of radioactivity.

Radioactivity measurements. Samples (0·3 ml.) of each of fractions 1–5, in duplicate, were placed on polythene disks of surface area 2 sq.cm. One drop of 0·2 % (w/v) cetyltrimethylammonium bromide (CTAB) and one drop of 1 % (w/v) polyvinyl alcohol were added to each sample and the preparations dried in vacuo. In this way films of less than 1 mg./sq.cm. were obtained and self-absorption was less than 5 %. The ^{14}C content of the dried preparations was estimated by use of an end-window Geiger-Muller tube and scaler in the conventional manner.

Investigation of major cell fractions. Fraction 1, the cold TCA-soluble fraction, was extracted with ether to remove TCA and chromatographed in the solvent systems described below. *Fraction* 2, ethanol-soluble protein, was hydrolysed in 6 N-HCl in sealed tubes for 16 hr. at 105°, and the hydrolysates, after removal of acid, run on two-dimensional chromatograms with *sec*-butanol + formic acid and phenol + ammonia as solvents. *Fraction* 3, the lipid fraction, was hydrolysed in 6 N-HCl for 48 hr. at 105° in sealed ampoules, the hydrolysates chilled and filtered, the filtrates evaporated to dryness in vacuo and, after removal of acid, chromatographed in *n*-butanol + morpholine solvent. *Fraction* 4, the nucleic acid fraction, was hydrolysed in 72 % (w/w) perchloric acid at 100° for 1 hr. and, after dilution with an equal volume of water, the hydrolysate was chromatographed in isopropanol + HCl solvent. *Fraction* 5 was treated in the same way as fraction 2.

Chromatography. One-dimensional descending and two-dimensional ascending chromatograms were run on Whatman no. 1 paper. Four solvents were used: *sec*-butanol, 70 % (v/v), formic acid, 10 % (v/v), water, 20 % (v/v); phenol crystals, 80 g., ammonium hydroxide (sp.gr. 0·880), 0·3 ml., water, 20 ml.; isopropanol, 68 % (v/v), hydrochloric acid (sp.gr. 1·18), 17·6 % (v/v), water, 14·4 % (v/v); *n*-butanol, 75 % (v/v), morpholine, 25 % (v/v). Radioautographs of chromatograms were prepared on Ilford (Industrial G) X-ray film.

Paper electrophoresis. Arginine, histidine and lysine were not well separated by two-dimensional chromatography but complete separation was obtained, after elution from chromatograms, by electrophoresis on paper buffered with 0·05 M-sodium carbonate + sodium bicarbonate buffer, pH 9·6; a potential of 800 V d.c. was applied for 2½ hr. (Dr R. Davies, private communication).

Isotopes. ^{14}C-labelled glucose, acetate and amino acids were obtained from the Radiochemical Centre, Amersham. Purines and pyrimidines were worked up from residues of *Chlorella* grown in $^{14}CO_2$ and supplied by the Radiochemical Centre, Amersham.

RESULTS

Nutritional requirements of Strigomonas oncopelti

Preliminary growth experiments showed that the complete defined medium supported the growth of organisms transferred from 3 % (w/v) peptone medium; further experiments were designed to determine the minimal growth requirements for *Strigomonas oncopelti.*

Amino acid requirements. The only amino acid present in the complete growth medium which was found to be essential for growth was methionine. In the absence of all other amino acids the concentration of methionine required for optimal growth was found to be 200 μg. DL-methionine/ml. Methionine could be replaced by: cystathionine alone; by homocysteine + a suitable 1-carbon source such as formate, glycine, serine or phosphoserine but not choline; by threonine + serine + cysteine; in this latter case the cysteine could be replaced by cysteine sulphinic acid but not by taurine, cysteic acid or an inorganic sulphur source.

Growth factor requirements. Thiamine, nicotinic acid and *p*-aminobenzoic acid were essential for growth when the only amino acid present was methionine.

Purine and pyrimidine requirements. No absolute requirement for purines or pyrimidines was found, but adenine (20 μg./ml.) could replace *p*-aminobenzoic acid.

Incorporation experiments

Utilization of acetate for synthesis. Acetate would not replace glucose as principle energy source for *Strigomonas oncopelti* growing in the minimal defined medium, but when ^{14}C-labelled acetate was added to a culture growing in this medium it became diluted with ^{12}C-acetate produced from glucose and was utilized for synthesis. To investigate the pathways by which such utilization occurred tracer amounts of labelled acetate were added to cultures in the logarithmic phase of growth; growth was allowed to continue for a further two to three generations after which the organisms were harvested, washed and fractionated as described.

Distribution of ^{14}C *from acetate in the major cell fractions.* Table 2 summarizes the results of typical experiments in which either $^{14}CH_3COOH$ or $CH_3{}^{14}COOH$ was used and shows that acetate was utilized for the synthesis of all the major classes of cellular compounds. The radioactivity of the cold-TCA-soluble material (fraction 1) was found mainly in free amino acids and some peptides; small amounts of labelled uracil were also found in this fraction.

Table 2. *Distribution of acetate-derived carbon in the major cell fraction of* Strigomonas oncopelti

Organisms were grown for 24 hr. in minimal defined medium supplemented with tracer amounts of either $^{14}CH_3COONa$ or $CH_3{}^{14}COONa$ before being fractionated as described in the text. Radioactivity of fractions is expressed as % total radioactivity fixed by whole organisms.

	^{14}C substrate	
	$^{14}CH_3COONa$	$CH_3{}^{14}COONa$
Fraction*	% total fixed radioactivity	
1. Cold TCA soluble	4	5
2. Ethanol soluble protein	3	4
3. Lipid	52	45
4. Nucleic acid	3	3
5. Protein	35	39
% recovery	97	96

* See text, p. 710.

About 50 % of the radioactive material synthesized by the organisms from acetate was extracted by 75 % (v/v) ethanol in water. Ether extraction separated the ethanol-soluble material into protein (fraction 2) and lipid (fraction 3) which contained most of the radioactivity. The nitrogenous constituents of the lipid fraction, separated after hydrolysis by chromatography, have been identified as serine and ethanolamine; no choline was detected by the phosphomolybdic acid method of Chargaff, Levine & Green (1948). These nitrogenous compounds contained no carbon derived from acetate.

Chromatograms of the hydrolysed hot-TCA-soluble fraction showed that ^{14}C derived from acetate was located only in the pyrimidines, cytosine, uracil and thymine.

Examination of the hydrolysed protein (fraction 5) by paper chromatography showed that both the methyl and carboxyl carbons of acetate were utilized in the synthesis of the following 13 amino acids: glutamic and aspartic acids, alanine, threonine, histidine, tyrosine, lysine, arginine, valine, proline, phenylalanine, leucine, isoleucine. No ^{14}C was detected in serine, glycine, cysteic acid or methionine, but the latter compound had been added to the growth medium. The same distribution of radioactivity was found in the amino acids of the ethanol-soluble protein (fraction 2). Table 3 summarizes the amounts of radioactivity found in various amino acids determined after their elution from two-dimensional chromatograms of protein hydrolysates; the results are expressed as % of the activity of glutamic acid. The relative proportions of some of the amino acids in the protein of *Strigomonas oncopelti* were estimated by the method of Abelson (1955) using ^{14}C randomly-labelled glucose and also by quantitative ninhydrin estimations (Cocking & Yemm, 1954). By using these values it is possible to calculate the relative specific activities of the amino acids labelled from acetate; these values are recorded in Table 3.

Table 3. *Distribution of acetate-derived carbon in the amino acids of* Strigomonas oncopelti

The protein fraction from organisms grown for 24 hr. in minimal defined medium supplemented with ^{14}C-labelled acetate was hydrolysed and the amino acids liberated were separated by two-dimensional chromatography. Amino acids were eluted from chromatograms for radioactivity determinations and the relative molar abundance of each was determined by quantitative ninhydrin estimations (Cocking & Yemm, 1954) or by the method of Abelson (1955). Results are expressed as activity, specific activity or molar abundance relative to 100 for glutamic acid in each case.

		Relative molar abundance determined by		
Amino acid	Relative activity	Abelson's method	Ninhydrin estimation	Relative specific activity
Glutamic acid	100	100	100	100
Arginine	63	60	—	105
Aspartic acid	48	73	72	66
Proline	37	31	37	110
Valine	34	68	62	52
Lysine	34	60	—	56
Phenylalanine*	29	—	—	—
Threonine	18	29	27	64
Tyrosine*	15	—	—	—
Alanine	8	80	85	10
Histidine*	6	—	—	—
Leucine + Isoleucine*	71	—	—	—
Glycine	0	—	62	0
Serine	0	—	52	0

* Values for the relative molar abundance of phenylalanine, leucine and isoleucine were not determined because a clear-cut chromatographic separation of these amino acids was not obtained with the solvent systems used. Values for the tyrosine and histidine content of protein are not included as these amino acids are subject to destruction during hydrolysis.

Utilization of randomly-labelled ^{14}C *amino acids.* In experiments designed to study the uptake of radioactive amino acids, organisms were grown in minimal defined medium supplemented with trace amounts (1 μg./ml. or less) of the given ^{14}C-labelled amino acid. Table 4 shows the distribution of radioactivity from a number of amino acids in the various cell fractions. In most cases the majority of the radioactivity was found in the protein. Glycine and serine contributed appreciable amounts of ^{14}C to the nucleic acid fraction and the addition of ^{12}C-adenine or guanine to the growth medium in the presence of ^{14}C-glycine decreased the incorporation of radioactivity into the nucleic acid. Radioactivity from glycine and serine was also incorporated into the nitrogenous constituents of the lipid fraction. Chromatographic examination of the protein hydrolysates obtained from these experiments gave some indication of

Table 4. *Distribution of* ^{14}C *from randomly-labelled amino acids among cellular fractions of* Strigomonas oncopelti

Organisms were grown for 24 hr. in minimal defined medium supplemented with tracer amounts of ^{14}C-labelled amino acids. Cell fractionation was carried out as described in the text (p. 710). Radioactivity of fractions is expressed as a percentage of the total radioactivity fixed by whole organisms.

	^{14}C-labelled amino acid added to minimal medium							
Fraction	Glutamic	Aspartic	Glycine	Glycine*	Serine	Leucine	Valine	Arginine
1. Cold TCA soluble	5	4	5	6	9	1	3	6
2. Ethanol-soluble protein	6	5	2	5	4	10	8	5
3. Lipid	12	12	6	7	15	0·5	0·5	2
4. Nucleic acid	10	8	38	16	12	0·5	0·5	2
5. Protein	59	58	45	60	51	78	77	83
% recovery	91	87	96	94	91	90	89	98

* In this experiment ^{12}C adenine (20 μg./ml.) was added to the minimal synthetic medium in addition to ^{14}C-labelled glycine.

Table 5. *Interconversion of amino acids by* Strigomonas oncopelti

Organisms were grown for 24 hr. in minimal defined medium supplemented with tracer amounts of randomly-labelled ^{14}C amino acids. Protein fractions of organisms were hydrolysed and chromatographed and radioautographs prepared from the chromatograms. The distribution of radioactivity on radioautographs is recorded: + +, +, (+), and −, represent very strong, strong, weak and no detectable labelling, respectively.

	^{14}C-labelled amino acid added to minimal medium						
	Aspartic	Glutamic	Glycine	Serine	Leucine	Valine	Arginine
Amino acids in protein hydrolysate	Relative degrees of ^{14}C-labelling of amino acids in protein hydrolysate						
Cysteic acid	−	−	+	+	−	−	−
Aspartic acid	+	+	−	−	−	−	−
Glutamic acid	+	+ +	−	−	−	−	(+)
Serine	−	−	+	+	−	−	−
Glycine	−	−	+	+	−	−	−
Threonine	+	+	−	−	−	−	−
Alanine	+	+	−	−	−	−	−
Tyrosine	+	+	−	−	−	−	−
Histidine	+	+	−	−	−	−	−
Lysine	+	+	−	−	−	−	−
Arginine	+	+ +	−	−	−	−	+ +
Proline	+	+ +	−	−	−	−	(+)
Valine	+	+	−	−	−	+	−
Phenylalanine	+	+	−	−	−	−	−
Leucine + Isoleucine	+	+	−	−	+	+	−

the ability of *Strigomonas oncopelti* to interconvert amino acids; the qualitative data obtained in this way are summarized in Table 5. An exogenous supply of ^{14}C-labelled glutamic acid was utilized by *S. oncopelti* for the synthesis of 12 amino acids and of these arginine and proline were found to have the same specific activity as the glutamic acid residues of the protein. On the other hand, radioactivity from ^{14}C-arginine was found almost entirely in the corresponding amino acid of the cell protein indicating that there was no appreciable conversion to glutamic acid, proline or other amino acids. The use of ^{14}C-glycine or serine showed that these two amino acids are readily interconverted by *S. oncopelti* and that they both contribute ^{14}C to cysteine, detected on chromatograms as cysteic acid. Exogenous ^{14}C-valine contributed carbon to leucine but the addition of ^{14}C leucine showed that the reverse reaction did not occur.

Utilization of labelled materials for nucleic acid synthesis. Table 6 summarizes the results of a number of experiments in which several ^{14}C-labelled substrates were used. With the exception of randomly-labelled glucose, which contributed carbon to all the components of nucleic acid, these compounds fall into two groups; the first group specifically labels purines, and includes glycine, serine and guanine; the second group labels pyrimidines and includes acetate, aspartic and glutamic acids and uracil. Thymine was not utilized by *Strigomonas oncopelti.*

Table 6. *Utilization of* ^{14}*C-labelled compounds for nucleic acid synthesis by* Strigomonas oncopelti

Organisms were grown for 24 hr. in minimal defined medium supplemented with tracer amounts of ^{14}C-labelled compounds. Nucleic acid fractions of organisms were hydrolysed and chromatographed, and radioautographs of the chromatograms prepared. The distribution of radioactivity on radioautographs is recorded: +, (+), and − represent strong, weak and no detectable labelling respectively.

	Nucleic acid component				
	Adenine	Guanine	Cytosine	Uracil	Thymine
^{14}C substrate	Relative degree of labelling				
Glucose	+	+	+	+	+
$^{14}CH_3COONa$	−	−	+	+	+
$CH_3{}^{14}COONa$	−	−	+	+	+
Aspartic acid	−	−	+	+	+
Glutamic acid	−	−	+	+	+
Glycine	+	+	−	−	−
Serine	+	+	−	−	−
Guanine	(+)	+	−	−	−
Uracil	−	−	+	+	+
Thymine	−	−	−	−	−

DISCUSSION

The data obtained from isotope incorporation experiments and from studies of the nutritional requirements of *Strigomonas oncopelti* permit some conclusions to be drawn concerning the biosynthetic pathways which function in this organism when it is growing in the simple defined medium used. The carbon of glucose and acetate can be utilized for the biosynthesis of all the amino acids except methionine, glycine, serine and cysteine. The work of a number of investigators (reviewed by Stekol, 1955) concerning the precursors of methionine allows the formulation of a pathway for methionine synthesis as follows:

$$\text{Threonine}\rightarrow\text{Homoserine}\xrightarrow[+\text{Cysteine}]{}\text{Cystathionine}\rightarrow\text{Homocysteine}\xrightarrow[+\text{Serine}]{}\text{Methionine}$$

That this pathway may be operating in *S. oncopelti* is suggested by the fact that serine + threonine + cysteine replace methionine in the defined growth medium and also that methionine may be replaced by cystathionine or by homocysteine + serine. Furthermore, this pathway appears to be reversible in *S. oncopelti* since acetate carbon was not detected in glycine or serine and the requirement of this organism for these two amino acids is met by methionine.

The results concerning purine and pyrimidine synthesis in *Strigomonas oncopelti* are in keeping with the relationships worked out for other organisms; purine synthesis involves the utilization of glycine and pyrimidine synthesis the utilization of aspartic acid. However, *S. oncopelti* differs from all other trypanosomid flagellates which have so far been studied in its ability to synthesise the purine nucleus. Thus *Crithidia fasciculata* (Nathan & Cowperthwaite, 1954), *Trypanosoma cruzi* (Citri & Grossowicz, 1955), *T. rhodesiense* (Williamson & Rollo, 1952) and *T. mega* (Bone & Steinert, 1956) appear to be incapable of synthesizing the purine ring.

The general pattern of results obtained from incorporation studies with $^{14}CH_3COOH$ and $CH_3{}^{14}COOH$ is similar to that reported by McQuillen & Roberts (1954), who studied the utilization of acetate by *Escherichia coli*. In *E. coli* acetate is utilized by three main pathways. One pathway leads to incorporation of acetate carbon into lipid, another to incorporation into leucine and by the third acetate enters the Krebs tricarboxylic acid cycle and contributes to the group of products derived from aspartic and glutamic acids. All these pathways would appear to function in *Strigomonas oncopelti*, and in addition acetate carbon is utilized for the synthesis of alanine, histidine, valine and the aromatic amino acids tyrosine and phenylalanine. Calculation of the specific radioactivities of the amino acids labelled with carbon derived from acetate throws some light on the inter-relationships between them. The specific activities of glutamic acid, arginine and proline are very nearly equal, suggesting a relationship between these amino acids in that they derive the same number of carbon atoms from acetate. There appears to be a similar relationship between aspartic acid and threonine. Furthermore, it was found that glutamic acid, arginine and proline had considerably higher specific activities than aspartic acid, threonine and lysine; as pointed out by McQuillen & Roberts (1954) for the case of *Escherichia coli*, this must be interpreted in terms of a tricarboxylic acid cycle rather than a dicarboxylic acid cycle involving the condensation of acetate to succinate, as the latter mechanism would result in the aspartic acid group having a higher specific activity than the glutamic acid group.

The lack of suitable defined growth media for parasitic flagellates may explain the fact that there have, as yet, been no other detailed studies of the biosynthetic capabilities of such organisms. Nevertheless, further work along these lines need not necessarily await the development of completely defined growth media as shown by the recent work of Grant & Fulton (1956) who studied the incorporation of ^{14}C-labelled glucose into the cellular components of *Trypanosoma rhodesiense* maintained in a salts + glucose + serum medium, and also by the work of Boné & Steinert (1956) who demonstrated the incorporation of adenine into nucleic acid and carbon from glycine and formate into protein of *T. mega* growing in a relatively complex medium.

REFERENCES

Abelson, P. H. (1955). Studies of biosynthesis in *Escherichia coli*. *Publ. Carneg. Instn*, no. 607, p. 28.

Boné, G. J. & Steinert, M. (1956). Isotopes incorporated in the nucleic acids of *Trypanosoma mega*. *Nature, Lond.* **178**, 308.

Chargaff, E., Levine, C. & Green, C. (1948). Techniques for the demonstration by chromatography of nitrogenous lipid constituents, sulfur-containing amino acids, and reducing sugars. *J. biol. Chem.* **175**, 67.

Citri, N. & Grossowicz, N. (1955). A partially defined culture medium for *Trypanosoma cruzi* and some other haemoflagellates. *J. gen. Microbiol.* **13**, 273.

Clausen, J. K. (1955). Observations on the carbohydrate metabolism of the flagellate *Strigomonas oncopelti*. *J. gen. Microbiol.* **12**, 496.

Cocking, E. C. & Yemm, E. W. (1954). Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, xii.

Gladstone, G. P. (1937). The nutrition of *Staphylococcus aureus*; nitrogen requirements. *Brit. J. exp. Path.* **18**, 322.

Grant, P. T. & Fulton, J. D. (1956). Glucose catabolism by strains of *Trypanosoma rhodesiense*. *Biochem. J.* **63**, 8*P*.

Lwoff, M. (1937). Aneurin as a growth factor for the trypanosomid flagellate *Strigomonas oncopelti*. *C.R. Soc. Biol., Paris*, **126**, 771.

McQuillen, K. & Roberts, R. B. (1954). The utilization of acetate for synthesis in *Escherichia coli*. *J. biol. Chem.* **207**, 81.

Nathan, H. A. & Cowperthwaite, J. (1954). Use of the trypanosomid flagellate, *Crithidia fasciculata*, for evaluating antimalarials. *Proc. Soc. exp. Biol., N.Y.* **85**, 117.

Nathan, H. A., Cowperthwaite, J., Carsted, K. W. & Hutner, S. H. (1955). In *Biochemistry and Physiology of Protozoa*. Ed. Hutner, S. H. & Lwoff, A., vol. 2, p. 35. New York: Academic Press Inc.

Newton, B. A. (1956*a*). A synthetic growth medium for the trypanosomid flagellate *Strigomonas* (*Herpetomonas*) *oncopelti*. *Nature, Lond.* **177**, 279.

Newton, B. A. (1956*b*). Biosynthetic capabilities of the parasitic flagellate *Strigomonas* (*Herpetomonas*) *oncopelti*. *J. gen. Microbiol.* **15**, viii.

Noguchi, H. & Tilden, E. B. (1926). Comparative studies of Herpetomonads and Leishmanias. *J. exp. Med.* **44**, 307.

Ryley, J. F. (1955). Studies on the metabolism of the protozoa. 4. Metabolism of the parasite flagellate *Strigomonas oncopelti*. *Biochem. J.* **59**, 353.

Stekol, J. A. (1955). Synthetic pathways of methionine, cysteine, and threonine. In *A Symposium on Amino Acid Metabolism*, p. 509. Ed. McElroy, D. W. & Glass, B. H. Baltimore: The Johns Hopkins Press.

Williamson, J. & Rollo, I. M. (1952). Stimulating effect of amino acids on the survival at 37° C of *Trypanosoma rhodesiense* in a serum-free synthetic medium. *Nature, Lond.* **170**, 376.

(*Received* 2 *July* 1957)

Newton, B. A. (1957). *J. gen. Microbiol.* **17**, 718–730

The Mode of Action of Phenanthridines: The Effect of Ethidium Bromide on Cell Division and Nucleic Acid Synthesis

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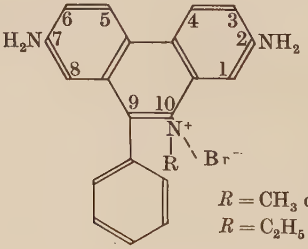
SUMMARY: The action of 2:7-diamino-9-phenyl-10-ethyl phenanthridinium bromide (ethidium bromide) on the parasitic flagellate *Strigomonas oncopelti* has been studied. The drug is irreversibly active only against growing organisms. Addition of the drug to cultures of organisms in the logarithmic phase of growth did not result in an immediate inhibition of growth but in a progressive decrease in growth rate; at least a doubling in number of organisms always occurred before multiplication finally ceased. During the period of growth in the presence of drug the deoxyribonucleic acid content of the organisms fell to half its normal value whilst the ribonucleic acid remained approximately constant. Conditions have been determined which permit the synthesis of nucleic acids and proteins by washed suspensions of *S. oncopelti* and the effect of ethidium bromide on these processes has been studied. The drug rapidly inhibits DNA synthesis whereas RNA and protein synthesis continue for a period of 2–3 hr. after the addition of drug. A study has been made of the uptake of ^{14}C-labelled ethidium bromide by organisms under conditions which will or which will not permit nucleic acid synthesis. The uptake of drug is of two types: (i) an initial rapid uptake which occurs in the absence of nucleic acid synthesis and which does not affect the subsequent growth of organisms; (ii) an additional uptake by growing organisms which appears to follow the course of RNA synthesis and which results, eventually, in an inhibition of growth.

Certain phenanthridinium compounds, originally synthesized by Morgan, Walls, Browning, Gulbranson & Robb (1938), possess trypanocidal properties (Browning, Morgan, Robb & Walls, 1938) and have been used with success against *Trypanosoma congolense* and *T. vivax* infections in cattle. Walls (1945) demonstrated that high trypanocidal activity in the phenanthridine series is a property of quaternary salts containing a primary amino group in the 7- and a phenyl group in the 9- position; the activity is much increased by the presence of a second amino group, thus 2:7-diamino-9-phenyl-10-methyl phenanthridinium bromide (dimidium bromide) and the 10-ethyl analogue (ethidium bromide) are particularly effective (Watkins & Woolfe, 1952; Woolfe 1952).



$R = CH_3$ dimidium bromide
$R = C_2H_5$ ethidium bromide

Up to the present time there have been few studies of the mode of action of these compounds. Lock (1950) examined the action of dimidium bromide upon *Trypanosoma congolense* and *T. bruceii* maintained *in vitro* and found that prolonged exposure under these conditions did not kill the trypanosomes, although some loss of infectivity was detected when drug-treated organisms were injected into animals. Chemical methods of analysis failed to show any uptake of drug by trypanosomes. Ormerod (1951*a*, *b*) showed, by histochemical techniques, that after 24 hr. contact with dimidium bromide, chromophilic granules containing ribonucleoprotein appeared in the cytoplasm of trypanosomes. He also observed that growth in the presence of dimidium bromide resulted in an increase in the number of multinuclear forms and a decrease in the number of dividing forms of trypanosomes; the motility of the organisms was unaffected. Ormerod obtained similar results with other trypanocidal drugs, including antrycide and suramin, and he postulated that these drugs prevent growth and cell division, but not nuclear division, by splitting cytoplasmic ribonucleoprotein into its constituent protein and nucleic acid. In support of this hypothesis he found that no antrycide could be detected in the nucleus of drug-treated trypanosomes by fluorescent microscopy whereas the cytoplasmic granules were strongly fluorescent, indicating the presence of drug in these structures. The slow action of these drugs, Ormerod suggested, was due to the slow rate of penetration into cells.

The antiviral and antibacterial activity of phenanthridines have been studied recently by Dickinson, Chantrill, Inkley & Thompson (1953) and by Seaman & Woodbine (1953); both groups of workers have observed that the action of these drugs can be annulled by addition of nucleic acid to the drug-containing medium.

Hawking (1953), reviewing current knowledge of trypanocidal compounds, suggested that further study of the mode of action of phenanthridines and related drugs might yield information about nucleic acid metabolism in general and the role of cytoplasmic ribonucleoprotein in particular; such studies have been delayed by the difficulties encountered in the cultivation and maintenance of pathogenic trypanosomes which are sensitive to the phenanthridine drugs. The trypanosomid flagellate *Strigomonas oncopelti* would seem to be ideally suited for the investigation of these problems; it can be readily cultivated in certain peptones (Lwoff, 1937) or in a simple chemically-defined medium (Newton, 1956) and its growth under these conditions is inhibited by low concentrations of ethidium bromide. The present paper describes the growth inhibitory action of ethidium bromide on *S. oncopelti* and its effect on the synthesis of nucleic acid and protein by this organism.

METHODS

Organism. The strain of *Strigomonas oncopelti* previously described (Newton, 1957) was used.

Conditions of culture. Stock cultures were maintained in 5 ml. volumes of 3 % (w/v) Evans Bacteriological Peptone containing 0·5 % (w/v) sodium chloride and 0·5 % (w/v) glucose, at an initial pH value of 7·4 (to be referred to as 'peptone glucose medium'). The medium was sterilized by autoclaving at 15 lb./sq.in. for 20 min. Cultures were incubated at 25° and subcultured every other day. Larger batches of organisms were grown in 50 ml. or 500 ml. of peptone glucose medium contained in either 150 ml. or 1-litre flasks; a 10 % (v/v) inoculum from a 15 hr. culture in the same medium was used and cultures were incubated with shaking in a water bath maintained at 25°.

Preparation of washed suspensions. Organisms were harvested by centrifugation, washed three times with a buffered salts solution containing (%, w/v): 0·9 NaCl; 0·4 KCl; 0·025 $Na_2HPO_4.2H_2O$; 0·008 KH_2PO_4; trace metals as described previously (Newton, 1957), and finally suspended in this solution to give a density of *c.* 10^8 organisms/ml. This suspension was diluted 1/10 during incubation under the various conditions to be described. Washed suspensions to be used for the study of nucleic acid and protein synthesis were prepared in the same way but under aseptic conditions.

Counts of organisms. In all experiments growth was estimated by counts; 1 ml. samples of cultures were fixed with 0·05 ml. 40 % (w/v) formaldehyde in water. Routinely four counts were made on each sample in a Neubauer haemocytometer, a minimum of 300 organisms being counted in each case. Ten counts/sample were made when cultures were being examined for synchronization of division.

Cytochemical techniques. Smears of organisms were prepared on microscope slides, dried in air and fixed by immersion in absolute methanol for 10 min. Preparations were then stained either with May-Grunwald and Giemsa dyes or with methyl green and pyronin G as described by Jacobson & Webb (1952). As a control for the staining of deoxyribonucleoprotein by the above methods, preparations were stained by Feulgen's method after 3 min. hydrolysis in N-HCl at 60°. In some cases fixed smears were treated with enzymes before staining; enzymes used were: crystalline protease-free ribonuclease, crystalline deoxyribonuclease (Sigma Chemical Co. U.S.A.) and crystalline trypsin (Worthington Biochemical Sales Co. U.S.A.). Enzyme solutions (0·1 %, w/v) were prepared in glass-distilled water, containing in the case of deoxyribonuclease, 0·075 M-$MgSO_4$. Preparations were incubated in enzyme solutions at 37° for different periods of time; in each experiment a control preparation was incubated in glass-distilled water alone.

Temperature-induced synchronous division in cultures of Strigomonas oncopelti. Partial synchronization of cell division was obtained by subjecting organisms, suspended in peptone glucose medium, to a series of controlled temperature shifts between 25° (the optimum growth temperature) and 5°. Suspensions containing 5×10^5 organisms/ml. were maintained at each temperature for 1 hr. and the temperature cycling was continued during 18 hr. Rapid temperature shifts were obtained by means of an apparatus similar to that described by Scherbaum & Zeuthen (1955).

Amino acid, purine and pyrimidine solutions. Solutions (1·2 % w/v) of glycine and of the L isomers of the following amino acids were prepared: L-aspartic and L-glutamic acids, L-arginine, DL-methionine, L-lysine, DL-threonine, L-proline, DL-tryptophan, L-tyrosine, L-cysteine, DL-serine, DL-phenylalanine, L-histidine, L-valine, DL-alanine, L-leucine and DL-isoleucine; each solution was adjusted to pH 7·0. Solutions (0·12 %, w/v) of each of the following purines and pyrimidines were prepared: adenine, guanine, xanthine, thymine, cytosine and uracil. All these solutions were diluted 1/100 in incubation mixtures.

Incubation mixtures. Incubation mixtures in 150 ml. conical flasks contained: 0·6 ml. 20 % (w/v) glucose, 0·1 ml. of each of the amino acid solutions, 0·1 ml. of each of the six purine and pyrimidine solutions, 7·3 ml. of the buffered salts solution described, 1·2 ml. of a washed suspension of *Strigomonas oncopelti* prepared as above, 0·5 ml. ethidium bromide solution; where any component was omitted an equal volume of buffered salts solution was added. Flasks were closed with cotton plugs and incubated with shaking at 25°. Before the addition of washed organisms and ethidium bromide, incubation mixtures were sterilized by autoclaving at 15 lb./sq.in. for 10 min., the cotton plugs being covered with grease-proof paper to prevent loss of contents by evaporation; suspensions of organisms and ethidium bromide were then added with the usual precautions to prevent contamination. Ten ml. from the incubation mixtures were transferred to 15 ml. centrifuge tubes at the end of an incubation period and rapidly cooled to 0°. The organisms were harvested by centrifugation, washed with cold buffered salts solution and analysed for protein and nucleic acid.

Protein and nucleic acid content of organisms. Suspensions of organisms after washing were precipitated with perchloric acid to a final concentration of 0·2 N. After standing for 1 hr. at 5° the precipitates were centrifuged down, washed twice with cold 0·2 N-perchloric acid and then extracted three times successively with 0·5 N-perchloric acid at 70° for 30 min.; 90–95 % of the total nucleic acid was extracted in the first two treatments and no further extraction took place after the third treatment. Samples of the combined extracts were taken for the determination of total nucleic acid content by measurement of absorption at 260 mμ. in a Beckman spectrophotometer (Model DU); samples were also taken for the determination of deoxyribose by the diphenylamine method of Burton (1956). The residue remaining after extraction with perchloric acid represented the main protein fraction of the organisms and was either suspended in 0·75 ml. 20 % (v/v) acetone in water for radioactivity determinations, or was digested by the Kjeldahl method under the conditions described by Chibnall, Rees & Williams (1943); the ammonia collected after distillation in a Markham apparatus (Markham, 1942) was determined colorimetrically with Nessler's reagent as described by Johnson (1941).

Radioactivity measurements. Samples (0·3 ml.) of protein fractions labelled with ^{14}C-glycine suspended in 20 % (v/v) acetone in water were placed on polythene disks of surface area 2 sq.cm.; 1 drop of 1 % (w/v) polyvinyl alcohol was added to each sample and the preparations dried *in vacuo*. In this way films of less than 1 mg./sq.cm. were obtained and self-absorption was less than 5 %. The ^{14}C content of dried preparations was estimated by use of an end window Geiger-Muller tube. In experiments designed to measure the uptake of ^{14}C-labelled ethidium bromide by *Strigomonas oncopelti*, organisms, after harvesting from a drug-containing medium, were washed three times with cold buffered salts solution and then suspended directly in 0·75 ml. 20 % (v/v) acetone in water. Samples (0·3 ml.) were then plated out as described above, but because the ^{14}C-labelled drug had a low specific activity (0·17 mc./g.) radioactivity measurements were made on a windowless gas-flow (methane) counter operating in the proportional range as described by Taylor & Sharpe (1951). Backgrounds and samples were counted sufficiently long to give a standard error of less than 5 % in all cases and counts were corrected for self absorption.

Isotopes. Glycine labelled with ^{14}C in the carboxyl position was obtained from the Radiochemical Centre, Amersham; dimidium and ethidium bromides, each labelled with ^{14}C in the 9 position in the phenanthridine ring, were the gift of Boots Pure Drug Co. Ltd., Nottingham.

RESULTS

Cytochemical observations

Growth of *Strigomonas oncopelti* for 12 hr. or more in the presence of ethidium bromide (15 μg./ml.) resulted in the appearance of refractile cytoplasmic granules which were readily seen in living organisms by phase contrast microscopy. In fixed preparations these granules were stained red by pyronin G, blue by May-Grunwald and Giemsa dyes and were Feulgen negative. Treatment of fixed smears of organisms with deoxyribonuclease did not affect the staining properties of the granules; treatment with ribonuclease resulted in a complete loss of basophilic material after 30 min. incubation at 37° and the residual granules appeared to be weakly eosinophilic. Complete digestion of the granules was achieved by treatment of fixed preparations with ribonuclease and trypsin for 30 min. at 37°. These findings are in agreement with the results of Ormerod (1951*b*) who examined the effect of dimidium bromide on *Trypanosoma equiperdum* and *T. rhodesiense* and they suggest that the granules resulting from drug treatment contain ribonucleoprotein. In contrast to Ormerod's results, no increase in the number of multinuclear forms of *Strigomonas oncopelti* resulted from growth in the presence of ethidium bromide.

Inhibition of division by ethidium bromide

Effect of drug concentration. Multiplication of *Strigomonas oncopelti* in peptone glucose medium was not immediately prevented by the addition of ethidium bromide; 70–80 % increases in number of organisms always occurred after the addition of 20–200 μg. ethidium bromide/ml. to a culture containing *c.* 5×10^6 organisms/ml. With less than 20 μg. ethidium bromide/ml. greater increases in numbers occurred before multiplication finally stopped (Fig. 1).

Reversibility of ethidium bromide inhibition. When multiplication of *Strigomonas oncopelti* had ceased in the presence of ethidium bromide, it was not restarted by removal of organisms to a drug-free medium. When organisms were transferred from a drug-containing medium to a drug-free medium at intervals before multiplication stopped, they continued to multiply, but at a decreased rate (Fig. 2). The growth rate of drug-treated organisms was related to the time they had been in contact with the drug and was found to remain constant for 3–4 generations, after which there was a gradual increase to the control growth-rate over a further two generations.

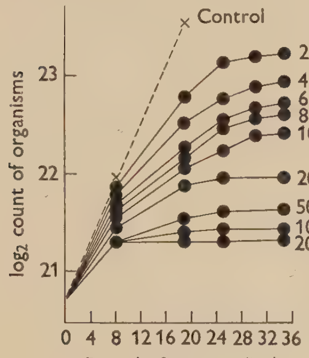


Fig. 1

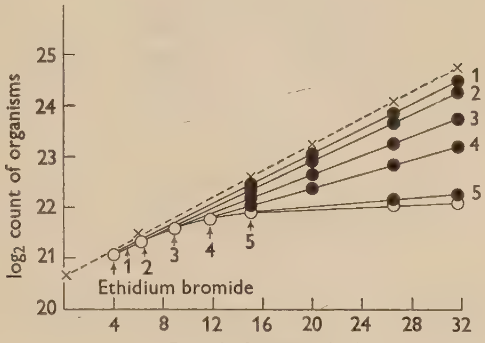


Fig. 2

Fig. 1. Effect of ethidium bromide concentration on the growth of *Strigomonas oncopelti*. Growth medium; 3 % (w/v) Evans Bacteriological Peptone containing 0·5 % (w/v) NaCl + 0·5 % (w/v) glucose. Growth temperature, 25°. Figures on graph indicate concentration of ethidium bromide in μg./ml. growth medium. Inoculum = 5×10^6 organisms/ml.

Fig. 2. Effect of ethidium bromide on the growth rate of *Strigomonas oncopelti*. (× – – – ×), control culture in peptone glucose medium; (○—○), +15 μg. ethidium bromide, added after 4 hr. of growth at 25°. Samples were removed from control and drug-treated cultures at the times indicated by arrows (1–5): the growth rate of the drug-treated organisms after washing and resuspending in a drug-free growth medium are recorded in curves 1–5. The growth rate of organisms removed from control cultures was not decreased by washing and resuspending in fresh growth medium.

Effect of ethidium bromide on non-dividing organisms

The effect of ethidium bromide on non-dividing organisms was examined under two conditions: (*a*) washed organisms suspended in a buffered salts solution containing 0·5 % (w/v) glucose and incubated at 25°; (*b*) organisms suspended in peptone glucose medium and incubated at 11°. Organisms could be incubated under either of these conditions for 10–12 hr. without any loss in motility or viability, but no division occurred nor was there any increase in the nucleic acid or protein content of organisms. Ethidium bromide (15 μg./ml.) was added to suspensions of organisms incubated under conditions (*a*) or (*b*). Samples were removed at intervals during a 10 hr. incubation period and the organisms washed free from drug, resuspended in peptone glucose medium and incubated at 25°; samples from control suspensions containing no drug were treated similarly. In all cases multiplication was estimated by counts of organisms. It was found that ethidium bromide (15 μg./ml.) was without effect on organisms incubated under either of the above conditions; no decrease in the growth rate of drug-treated organisms, compared with control organisms, occurred after 10 hr. contact with the drug.

Effect of ethidium bromide on synchronously dividing organisms

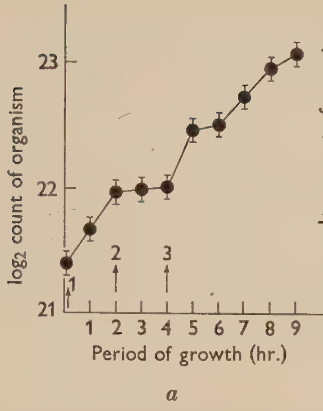
The slow action of ethidium bromide and the doubling in number of organisms which occurs before the multiplication of *Strigomonas oncopelti* is stopped by this drug might be due to differences in drug sensitivity of organisms at different stages in the division cycle. Only a small percentage of the total population of a culture of actively growing organisms will be in a particular phase of cell division at any one time; thus if organisms are only sensitive to ethidium bromide at one stage in the division cycle the majority of a population would be expected to divide once after the addition of drug to a culture. By using cultures in which cell division was partially synchronized it has been possible to examine the effect of ethidium bromide on a large number of organisms in approximately the same stage of cell division.

Partial synchronization of cell division in cultures of *Strigomonas oncopelti* was induced as described above. Fig. 3*a* shows a typical stepped growth curve obtained by these methods; the initial increase in count of organisms corresponds to the division of *c.* 70 % of the total population in a period of time equal to about one-quarter of a normal generation time (7·5–8 hr.).

In experiments designed to test the effect of ethidium bromide on organisms in different stages of division, a 400 ml. volume of peptone glucose medium containing *c.* 10^6 organisms/ml. growing in partial synchrony was divided into 50 ml. samples; each sample was placed in a 150 ml. conical flask and incubated at 25°. At intervals of 2 hr. ethidium bromide (final concentration 20 μg./ml.) was added to the contents of one flask and the incubation continued for a further 2 hr. The organisms were then harvested, washed and resuspended in drug-free peptone glucose medium and re-incubated at 25°. Multiplication was estimated by counts of organisms. The contents of one flask to which no drug had been added were harvested at the same time as samples of drug-treated organisms and were treated similarly. The curves obtained for the growth of drug-treated organisms (Fig. 3*b*) show that 2 hr. contact with ethidium bromide resulted in the same decrease in growth rate, regardless of the stage in cell division at the time of contact with the drug. These results show that the slow action of ethidium bromide is not due to variations in the drug sensitivity of organisms at different stages in the division cycle.

Changes in the nucleic acid content of ethidium bromide-treated organisms. Table 1 records the changes in nucleotide, ribonucleic acid and deoxyribonucleic acid content of organisms during asynchronous growth in the presence or absence of ethidium bromide. When the drug (final concentration, 20 μg./ml.) was added to a culture of initial density 2×10^6 organisms/ml. multiplication continued for 16 hr. and the count of organisms approximately doubled before multiplication ceased; during this time the deoxyribonucleic acid content of organisms fell progressively to half its normal value, whereas the nucleotide and ribonucleic acid content changed little.

Effect of ethidium bromide on nucleic acid and protein synthesis by washed suspensions of Strigomonas oncopelti. Gale & Folkes (1953), when studying



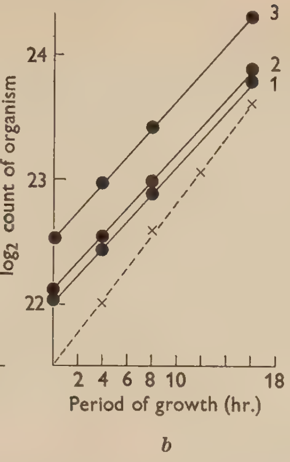


Fig. 3*a*. Partial synchronization of cell division in cultures of *Strigomonas oncopelti*. A stepped growth curve obtained by subjecting organisms, suspended in peptone glucose medium (5×10^5 organisms/ml.), to a series of controlled temperature shifts between 25° and 5°: suspensions were held at each temperature for 1 hr.; total period of temperature shifts, 18 hr.; final growth temperature 25°. Each point on the graph is the mean of 10 counts and the bars through these points represent the standard error of the mean of these determinations.

Fig. 3*b*. Effect of ethidium bromide on organisms dividing in partial synchrony. Ethidium bromide (20 μg./ml.) added at 2 hr. intervals to 50 ml. samples of a culture containing organisms dividing in partial synchrony. Arrows in Fig. 3*a* indicate the points on the growth curve at which drug was added. After 2 hr. contact with the drug organisms were harvested, washed and resuspended in drug-free peptone glucose medium. Growth was estimated by counts of organisms at intervals during 14 hr. of incubation at 25°.

Table 1. *Changes in the ribonucleic acid, deoxyribonucleic acid and nucleotide content of* Strigomonas oncopelti *growing in the presence of ethidium bromide*

Growth medium; peptone glucose. Growth temp. 25°. Inoculum $= 5 \times 10^6$ organisms/ml. Samples were taken at intervals from a control culture (C) and an ethidium bromide (20 μg./ml.)-containing culture (E); organisms were harvested, washed and their nucleic acid content determined as detailed in the text. The figures indicate changes in nucleotide, ribonucleic acid (both expressed as log I_0/I at 260 mμ./10^9 organisms) and deoxyribonucleic acid (estimated by the diphenylamine method and expressed as log I_0/I at 600 mμ./10^9 organisms) content of organisms growing in the presence or absence of drug during 24 hr.

Time of sampling (hr.)	log I_0/I at 260 mμ./10^9 organisms				log I_0/I at 600 mμ./10^9 organisms	
	Nucleotide		Ribonucleic acid		Deoxyribonucleic acid	
	C	E	C	E	C	E
0	3·6	3·5	150	160	0·59	0·65
2	3·0	3·2	163	141	0·55	0·58
4	3·0	3·4	149	157	0·51	0·54
6	3·1	2·9	189	152	0·60	0·47
8	3·2	3·1	176	142	0·60	0·40
12	3·0	3·1	171	136	0·51	0·41
24	2·9	3·0	157	141	0·58	0·31

nucleic acid and protein synthesis by washed suspensions of *Staphylococcus aureus*, found that there was an increase in the nucleic acid content of the organisms when they were incubated in the presence of glucose, a mixture of amino acids, purines and pyrimidines; little or no formation of nucleic acid occurred in the absence of amino acids or glucose. Similar results have been obtained for *Strigomonas oncopelti*; when washed organisms were incubated at 25° for 5 hr. with glucose, purines, pyrimidines and 18 amino acids the ribonucleic acid and protein content of the suspension increased by 70–80 % and the deoxyribonucleic content by 35–45 % (Fig. 4). The addition of ethidium bromide (20 µg./ml.) to a washed suspension incubated under these conditions resulted in a rapid inhibition of deoxyribonucleic acid synthesis whilst protein and ribonucleic acid synthesis continued for 2–3 hr. (Fig. 5).

Uptake of ^{14}C-labelled ethidium bromide by Strigomonas oncopelti

Uptake by organisms growing in peptone glucose medium. Fig. 6 shows that the uptake of ^{14}C-labelled ethidium bromide by organisms growing in a peptone glucose medium continued throughout the period of growth in the presence of the drug. A considerable uptake (*c.* 3 µg. ethidium/10^8 organisms) occurred during the lag phase of growth, during which time there was no increase in number of organisms.

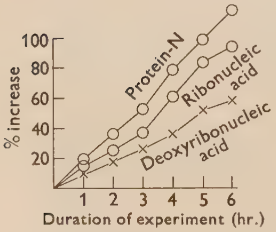


Fig. 4

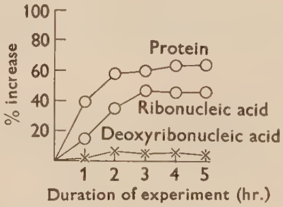


Fig. 5

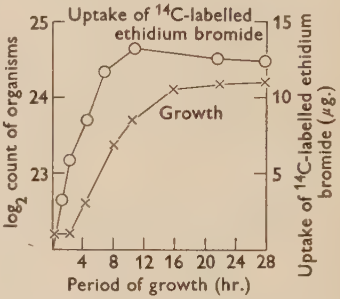


Fig. 6

Fig. 4. Synthesis of ribonucleic acid, deoxyribonucleic acid, and protein by washed *Strigomonas oncopelti*. Washed organisms suspended in buffered salts solution (pH 7·2) containing glucose, 18 amino acids, purines and pyrimidines; incubation temp. 25°.

Fig. 5. Effect of ethidium bromide (15 µg./ml.) on ribonucleic acid, deoxyribonucleic acid and protein synthesis by *Strigomonas oncopelti*. Washed organisms suspended in buffered salts solution (pH 7·2) containing glucose, 18 amino acids, purines and pyrimidines; incubation temp. 25°.

Fig. 6. Uptake of ^{14}C-labelled ethidium bromide by *Strigomonas oncopelti*. Growth in peptone glucose medium. Growth temp. 25°.

Release of drug from organisms. To determine whether ethidium bromide which had been bound by organisms was released again during multiplication in a drug-free medium, organisms were suspended in peptone glucose medium containing 10 µg. ^{14}C-labelled ethidium bromide/ml. and incubated at 25° for 4 hr. Organisms were then harvested, washed and resuspended in drug-free peptone glucose medium and incubated at 25° for a further 22 hr. Samples were taken at intervals during this second incubation for counting the number of organisms and for the determination of ^{14}C-labelled drug bound to the organisms. Table 2 shows that there was no significant release of ^{14}C-labelled drug from the organisms during 22 hr. of growth in a drug-free medium.

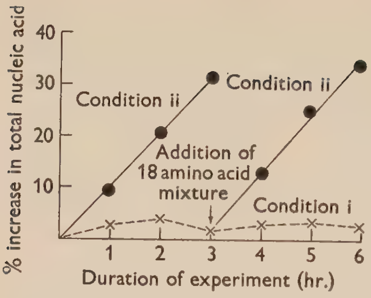
Table 2. *Uptake of* ^{14}C-*labelled ethidium bromide by* Strigomonas oncopelti. *Effect of growth in a drug-free medium after an initial period of growth in the presence of drug*

Organisms were grown for 4 hr. in peptone glucose medium containing 10 μg. ^{14}C-labelled ethidium bromide/ml., washed three times with drug-free peptone glucose medium and resuspended in this medium. Organisms from samples taken at intervals during a 22 hr. period of incubation at 25° were harvested, washed, suspended in 20 % (v/v) acetone in water and dried on polythene planchettes for radioactivity determinations in a windowless gas-flow (methane) counter.

Time after removal from ^{14}C-ethidium bromide (hr.)	Organisms $\times 10^{-4}$/ml.	Radio-activity/sample (counts/min.)
0	447	48
5	605	43
10	1468	45
22	3048	43

Uptake of ^{14}C-*labelled ethidium bromide by washed suspensions.* This was studied under two conditions: (i) washed organisms suspended in buffered salts solution containing glucose, purines and pyrimidines; (ii) as condition (i) but with the addition of a mixture of 18 amino acids. Under condition (i) organisms synthesized little or no nucleic acid during 6 hr. of incubation at 25°; under condition (ii) a linear increase in the nucleic acid content of organisms occurred. Addition of a mixture of 18 amino acids to organisms incubated under condition (i) initiated nucleic acid synthesis (Fig. 7*a*). ^{14}C-labelled ethidium bromide (final concentration, 10 μg./ml.) was added to organisms incubated under condition (i) and samples for radioactivity determinations were taken at intervals during a 6 hr. incubation period at 25°. It was found that drug was rapidly bound by organisms during the first 30 min. of the incubation period (*c.* 3 μg./10^8 organisms) but no more drug was bound during the next 5·5 hr.; addition of a mixture of 18 amino acids to the suspension resulted in a 40 % increase in the amount of ^{14}C-labelled ethidium bromide bound by the organism. Fig. 7*b* records these results; the ^{14}C-labelled drug bound by organisms is expressed as a percentage of the amount bound during 30 min. incubation under condition (i). The uptake of radioactive drug by organisms incubated under condition (ii) is also shown in Fig. 7*b*.

The effect of dimidium bromide on division and nucleic acid synthesis in Strigomonas oncopelti. Similar experiments to those already described were performed with dimidium bromide; the results showed that this drug has an effect similar to ethidium bromide on the division of *Strigomonas oncopelti* and on the synthesis of nucleic acid and protein by this organism.



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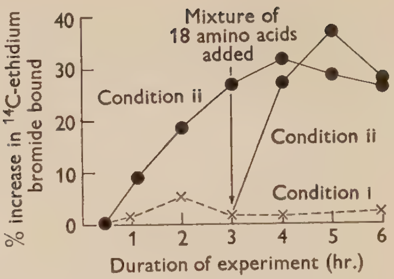

b

Fig. 7*a*. Synthesis of nucleic acid by washed suspensions of *Strigomonas oncopelti*. Condition i, organisms suspended in buffered salts solution containing glucose, purines and pyrimidines. Condition ii, as condition i but with the addition of a mixture of 18 amino acids. Incubation temp. 25°.

Fig. 7*b*. Uptake of ^{14}C-labelled ethidium bromide by washed suspensions of *Strigomonas oncopelti* incubated under condition i or condition ii. The uptake of radioactive drug expressed as % of increase in the amount bound by organisms during 30 min. incubation under condition i. Incubation temp. 25°.

DISCUSSION

Cytochemical studies led Ormerod (1951*b*) to propose that dimidium bromide inhibited the growth of trypanosomes by splitting cytoplasmic ribonucleoprotein into its constituent ribonucleic acid and protein. This hypothesis does not provide an adequate explanation of the results described in the present paper; it would be expected that a compound which caused the disorganization of nucleoprotein by the cleavage of electrostatic bonds would be active against non-growing organisms, but this was found not to be the case.

It is possible to advance a number of explanations for the slow action of ethidium bromide and for the approximate doubling in number of organisms which occurs before the multiplication of *Strigomonas oncopelti* is stopped by this drug. First, variations in drug sensitivity of organisms during a cell division cycle could produce such an effect; the results of experiments with cultures of synchronously dividing organisms have eliminated this possibility. Secondly, Ormerod (1951*b*) suggested that a slow penetration of dimidium bromide into organisms might explain the slow action of the drug. This explanation seems unlikely for the case of *S. oncopelti* since non-growing but actively metabolizing organisms (i.e. organisms incubated under the conditions described which do not permit multiplication or net synthesis of either protein or nucleic acid) can be exposed to both ethidium and dimidium bromide for long periods without any loss of viability. A third possibility is that the drugs penetrate into organisms rapidly but are not inhibitors of multiplication until they become bound on to, or incorporated into, a cellular component which only becomes available for this combination during growth. Such an explanation is compatible with some of the results obtained in the present work, but it does not explain the rapid binding of ^{14}C-labelled drugs by non-growing organisms. The results described in the present paper can be explained by postulating two types of combination between drug and organisms: a rapid combination with 'primary binding sites' which does not interfere with growth and cell division and which occurs in both non-growing and growing organisms, and then a slower combination with 'secondary binding sites' which become available to the drug during growth. It is this secondary uptake which results in a progressive decrease in the growth rate of organisms. The nature of these binding sites is not yet known, but there is some evidence to suggest that nucleic acid may be involved: the drugs can combine with, and are inactivated by, nucleic acid (Brownlee *et al.* 1950; Seaman & Woodbine, 1953); cytochemical studies (Ormerod, 1951*b*) suggest that the chromophilic granules which appear in the cytoplasm of drug-treated organisms contain ribonucleic acid, protein and bound drug, and the present work indicates that there is a close relationship between the combination of drug with 'secondary binding sites' and ribonucleic acid synthesis.

Inactivation of ribonucleic acid at the time of its synthesis might explain the observed progressive decrease in the growth rate of drug-treated organisms, since a correlation between the growth rate and ribonucleic acid content of cells has now been established for a number of microorganisms (Casperson & Brand, 1941; Jeener & Brachet, 1944; Caldwell, Mackor & Hinshelwood, 1950; Gale & Folkes, 1953). However this would not explain the rapid inhibition of deoxyribonucleic acid synthesis which occurs before the inhibition of either ribonucleic acid or protein synthesis, unless an active ribonucleic acid or ribonucleoprotein is in some way involved in deoxyribonucleic acid synthesis. Such a conclusion cannot be drawn from the experimental evidence available at the present time and a direct interaction between ethidium bromide and deoxyribonucleic acid cannot be excluded. It is hoped that further study of the mechanism of action of phenanthridine drugs may give some information about the interrelationships existing between deoxyribonucleic acid, ribonucleic acid and protein synthesis.

I wish to thank Dr D. H. Marrian of the Department of Radiotherapeutics, Cambridge, for making radioactivity measurements which involved the use of a windowless, gas-flow, counter.

REFERENCES

Browning, C. H., Morgan, G. T., Robb, J. M. V. & Walls, L. P. (1938). The trypanocidal action of certain phenanthridinium compounds. *J. Path. Bact.* **46**, 203.

Brownlee, G., Goss, M. D., Goodwin, L. G., Woodbine, M. & Walls, L. P. (1950). The chemotherapeutic action of phenanthridine compounds. *Brit. J. Pharmacol.* **5**, 261.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.

Caldwell, P. C., Mackor, E. L. & Hinshelwood, C. (1950). The ribose nucleic acid content and cell growth of *Bact. lactis aerogenes*. *J. chem. Soc.* p. 3151.

Casperson, T. & Brand, K. (1941). Nucleotidumsatz und Wachstum bei Presshefe. *Protoplasma*, **35**, 507.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354.

DICKINSON, L., CHANTRILL, B. H., INKLEY, G. W. & THOMPSON, M. J. (1953). The antiviral action of phenanthridinium compounds. *Brit. J. Pharmacol.* **8**, 139.

GALE, E. F. & FOLKES, J. P. (1953). The assimilation of amino acids by bacteria. 14. Nucleic acid and protein synthesis in *Staphylococcus aureus. Biochem. J.* **53**, 483.

HAWKING, F. (1953). Recent advances in the chemotherapy of protozoal infections. *Symposium. Growth Inhibition and Chemotherapy*, p. 88. *6th Congr. Int. Microbiol., Rome.*

JACOBSON, W. & WEBB, M. (1952). The two types of nucleoprotein during mitosis. *Exp. Cell Res.* **3**, 163.

JEENER, R. & BRACHET, J. (1944). Recherches sur l'acide ribonucleiques des levures. *Enzymologia*, **11**, 222.

JOHNSON, M. J. (1941). Isolation and properties of a pure yeast polypeptidase. *J. biol. Chem.* **137**, 575.

LOCK, J. A. (1950). The chemotherapeutic action of phenanthridine compounds. 4. Activity *in vitro. Brit. J. Pharmacol.* **5**, 398.

LWOFF, M. (1937). L'aneurine, facteur de croissance pour le flagelle trypanosomide, *Strigomonas oncopelti. C.R. Soc. Biol., Paris*, **126**, 771.

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. *Biochem. J.* **36**, 790.

MORGAN, G., WALLS, L. P., BROWNING, C. H., GULBRANSON, R. & ROBB, J. M. V. (1938). Researches in the phenanthridine series. 5. The colour and antiseptic properties of quaternary salts. *J. chem. Soc.* 389.

NEWTON, B. A. (1956). A synthetic growth medium for the trypanosomid flagellate *Strigomonas* (*Herpetomonas*) *oncopelti. Nature, Lond.* **177**, 279.

NEWTON, B. A. (1957). Nutritional requirements and biosynthetic capabilities of the parasitic flagellate *Strigomonas oncopelti. J. gen. Microbiol.* **17**, 708.

ORMEROD, W. E. (1951*a*). The mode of action of antrycide. *Brit. J. Pharmacol.* **6**, 325.

ORMEROD, W. E. (1951*b*). A study of basophilic inclusion bodies produced by chemotherapeutic agents in trypanosomes. *Brit. J. Pharmacol.* **6**, 334.

SCHERBAUM, O. & ZEUTHEN, E. (1955). Temperature induced synchronous division in the ciliate protozoan *Tetrahymena pyriformis* growing in synthetic and proteose peptone media. *Exp. Cell Res.* (Suppl.), **3**, 312.

SEAMAN, A. & WOODBINE, M. (1953). Some aspects of the antibacterial effects of phenanthridine compounds. *Atti VI Congr. int. Microbiol.*, **1**, 636.

TAYLOR, D. & SHARPE, J. (1951). Nuclear particle and radiation detectors. *Proc. Inst. Elect. Engrs.* **98**, 174.

WALLS, L. P. (1945). Researches in the phenanthridine series. 6. The relationship between structure and trypanocidal properties. *J. chem. Soc.* 294.

WATKINS, T. I. & WOOLFE, G. (1952). Effect of changing the quaternizing group on the trypanocidal activity of dimidium bromide. *Nature, Lond.* **169**, 506.

WOOLFE, G. (1952). The trypanocidal activity of phenanthridine compounds. *Ann. trop. Med. Parasit.* **46**, 285.

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The Uptake of Amino Acids During the Terminal Bacteraemia in Guinea Pigs Infected with *Bacillus anthracis*

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SUMMARY: As part of an attempt to study the metabolism of *Bacillus anthracis in vivo* and its connexion with virulence, the utilization of amino acids by this pathogen was examined whilst it was growing in the blood of guinea pigs during the terminal phases of anthrax. Under these conditions, *B. anthracis* used relatively large quantities of glutamine, threonine, tryptophan and glycine, whereas histidine, lysine, tyrosine, phenylalanine, methionine and alanine did not appear to be taken up in appreciable quantities.

When *Bacillus anthracis* grows *in vivo* it has properties different from those it has when it grows *in vitro* (Smith & Keppie, 1955). This means that the metabolism of the pathogen when growing in the tissues of the host is different from that of the same organism growing in artificial culture. An attempt has therefore been made to study some aspects of the metabolism of this organism when growing *in vivo*. As the basis for these studies we have had the benefit of the observations of Keppie, Smith & Harris-Smith (1955) on the final bacteraemia of anthrax in the guinea pig. They showed that, although guinea pigs varied in their initial resistance to infection with *B. anthracis*, once the final bacteraemia had started (*c.* 10–12 hr. before death) there was a relatively constant growth rate of bacteria in the blood as well as a remarkably constant relationship between bacterial growth and the survival time of the animal. We have made our examination of the metabolism of *B. anthracis* during this final bacteraemia in the guinea pig.

This paper deals with the utilization of amino acids by the pathogen. The object has been to determine changes in the free amino acid content of infected guinea-pig plasma at successive stages of the bacteraemia, and to verify that, as far as possible, recognition of any utilization which might have occurred was not obscured by compensatory mechanisms in the guinea-pig tissues. In an attempt to fulfil the latter condition a number of precautions were taken. Furthermore, it was verified that a similar pattern of utilization occurred when the infected plasma was withdrawn from the guinea pig and incubated *in vitro*. In many respects this test was adequate but it cannot be relied on absolutely since it is already apparent that the metabolism (though not necessarily the amino acid metabolism) of *Bacillus anthracis* is different *in vivo* from that which occurs *in vitro*.

METHODS

Collection of samples of plasma from infected guinea pigs at selected stages of the final bacteraemia

Guinea pigs (650 ± 50 g.) were infected with *Bacillus anthracis* strain N.P.; on the third day the terminal bacteraemia was detected and followed during the last 10–12 hr. by the methods described by Keppie *et al.* (1955). The guinea pigs were allowed water but no food during the experiments to minimize fluctuations in their free amino acid pool. During this period the bacterial count rises from approx. $0\cdot2\times10^6$ chains/ml. blood to 1×10^9 chains/ml. in 12 hr. It would have been opportune to allow this maximal growth of the organism to take place before taking the final plasma sample, so that the amino acid uptake was as large as possible. However, preliminary experiments showed this to be impossible. Determinations of the total α-carboxylamino-N of plasma samples by the method of Woiwod (1949) showed that during the last 3 hr. of the bacteraemia a large influx of amino acids into the blood occurred, probably as a result of tissue breakdown; the total amino acid content of the plasma rose four to fivefold. In addition, haemolysis became extensive in the last 3 hr. of life. For these reasons the plasma was taken for analysis at an earlier time. It was impossible to obtain from one guinea pig sufficient plasma for subsequent amino acid analysis, so the plasma of two similar guinea pigs was collected and pooled for analysis. Two guinea pigs were selected whose blood had bacterial counts within the range $0\cdot2$–$0\cdot8\times10^6$ chains/ml. Blood (3 ml.) was removed from each animal by cardiac puncture with a fine needle. This was pooled, heparinized (10 i.u. heparin/ml.) and centrifuged at 150 ***g*** for 30 min. at 0–2° to remove blood cells. The plasma was sterilized by filtration through Millipore filters and stored at $-15°$ until analysed. The two animals were allowed to live and the developing bacteraemia was followed by regular sampling from an ear vein. When the blood contained 1–$1\cdot5\times10^8$ chains/ml., 5 ml. blood was removed by cardiac puncture and treated as described above. The plasma was centrifuged at 8000 ***g*** for 20 min. at 0–2° to remove the large numbers of *Bacillus anthracis* which otherwise would have interfered with filtration.

The parallel growth in vitro *of* Bacillus anthracis *in plasma withdrawn from infected guinea pigs*

Two infected guinea pigs, which had the same bacterial count in the blood ($0\cdot2$–$0\cdot8\times10^6$ chains/ml.) as those used above, were selected. The maximum quantity of blood (*c.* 12 ml.) was removed by cardiac puncture from each animal, heparinized (10 i.u. heparin/ml.), pooled and centrifuged at 150 ***g*** for 30 min. to sediment blood cells. All operations subsequent to removing the blood from the animal were carried out at 37° and under aseptic conditions. The plasma had the same bacterial content as the whole blood, but only 0·3 % and 2·0 % of the original red blood cells and white blood cells, respectively. The plasma was treated as follows:

(i) One-quarter of the total volume of plasma was immediately cooled to 4°, sterilized by filtration and stored at $-15°$ until required for analysis.

(ii) Half the total volume of plasma was immediately transferred to a warmed Warburg flask and shaken (100 strokes/min.) in a bath at 37°; a slow stream of moist warm air containing 5 % (v/v) CO_2 was passed over the culture. The growth of the organisms was followed by counting stained smears, prepared as those from guinea pigs, until the total count of cells/ml. plasma had increased to that corresponding to the final count of the guinea-pig blood in the *in vivo* experiments. In making this comparison, account was taken of the fact that *in vitro* the mean chain length 7·0 (S.D. 3·8) cells/chain was longer than that *in vivo*, 4·2 (S.D. 1·6) cells/chain. After the required amount of growth had taken place, the infected plasma was removed from the Warburg flask, cooled rapidly to 4°, centrifuged at 12,000 *g* for 20 min. to remove the bulk of the organisms, and filtered through Millipore filter. The filtered plasma was stored at −15° until required for analysis.

Under these conditions the growth of the organism occurred at a somewhat slower rate than that observed *in vivo* and a period of 1–2 hr. longer was needed to attain a similar count. An adequate carbohydrate source was present throughout the growth; determinations of reducing sugar by the method of Hagedorn & Jensen (1923) as modified by Fujita & Iwatake (1931) showed that after growth, 60 % of the original blood sugar was still present. The organisms were as capsulated as those growing *in vivo* but were perhaps less basophilic. In all experiments no contaminants were detected and evaporation from the Warburg vessels was negligible.

(iii) To assess more accurately the change in concentration of amino acids brought about by the growth of the organism, it was necessary to determine the changes produced in the plasma by processes other than those related to the presence of the organism. For this reason, the remaining plasma was freed from bacteria by filtration and incubated in a Warburg flask under the same conditions as for the infected plasma. It was then rapidly cooled and stored at −15° until required for analysis. In Table 2 the average percentage change in concentration of each amino acid has been calculated, first (see (*a*) in Table 2) on the basis of the initial concentration, and secondly (see (*b*) in Table 2) on the basis of the concentration after incubation in the absence of bacteria. It will be noted from Table 2 that with the exception of serine and methionine both calculations yielded similar results.

The rate of liberation of amino acids in this period of incubation was not extensively altered by extracellular processes resulting from the growth of the organism. When some of the plasma, used in (ii) above, was further incubated in the absence of bacteria, the rate of release of individual amino acids was not significantly greater than that shown in Table 2.

Amino acid analysis of plasma

Removal of protein. This was carried out by the method of Hier & Bergeim (1945) by using tungstic acid. After addition of the precipitant to the plasma (1 ml.), the mixture was allowed to stand at 4° overnight. The precipitate was removed by centrifugation and the supernatant (*c*. 3 ml.) brought to pH 7 with a trace of dilute NaOH.

Microbiological assays. These were carried out on an ultramicro scale by methods similar to those of Agren (1950). All assays were done in triplicate, the total volume in each tube being 200 μl. For each assay, a curve was constructed with the results from five dilutions of a standard solution of amino acid. The concentration of each amino acid in a sample equivalent to 33 μl.

original plasma (i.e. 100 μl. of the filtrate after tungstic acid precipitation) was determined.

Methionine, phenylalanine, tyrosine, tryptophan, threonine, serine, glycine and histidine were assayed with *Leuconostoc mesenteroides* P. 60 in the medium of Henderson & Snell (1948), and cysteine with the same organism in the medium of Steele, Sauberlich, Reynolds & Baumann (1949). Valine and lysine were assayed by using *Streptococcus faecalis* R again in the medium of Henderson & Snell and glutamic acid (+ glutamine) with *Lactobacillus arabinosus* 17–5 in the medium of Dunn, Camien, Rockland, Shankman & Goldberg (1944). Recoveries of 92–118 % were obtained by these methods when standard quantities of amino acids were added to normal guinea-pig plasma before tungstic acid precipitation.

Paper chromatography. Significant changes in the concentration of leucine + isoleucine, alanine, glutamic acid + glutamine were adequately detected by two-dimensional paper chromatography. After removal of protein, the plasma samples were de-salted in the apparatus of Consden, Gordon & Martin (1947). The solutions of free amino acids were neutralized with ammonia and evaporated in a vacuum desiccator. Samples, equivalent to 0·25 ml. plasma were loaded on to paper chromatograms which were developed in (*a*) phenol saturated with water; (*b*) butanol (4 vol.) + acetic acid (1 vol.) + water (4 vol.), by the capillary ascent method (Williams & Kirby, 1948). After evaporation of solvent at room temperature, the chromatograms were sprayed with a ninhydrin solution (0·2 %, w/v, ninhydrin in 95 %, v/v, butanol in water), heated at 95° for 5 min. and examined visually after standing a further 1 hr. at room temperature. It was shown by experiment that under these conditions, a change of 25 % in concentration of the appropriate amino acids could be detected.

Proline estimation. This was carried out by the method of Troll & Lindsley (1955) on 0·08 ml. samples of the deproteinized plasma. An 88 % recovery was obtained by this method when proline was added to normal guinea-pig plasma before tungstic acid precipitation.

A satisfactory method of estimating arginine and aspartic acid in the small samples of plasma available was not obtained and observations on the utilization of these amino acids were not made.

RESULTS

Table 1 summarizes the results of amino acid analyses before and after *in vivo* growth of *Bacillus anthracis.* Clearly glutamine, glycine, threonine and tryptophan were utilized by the pathogen. With the exception of serine, which remained almost unaltered, the concentration of all the other amino acids in the plasma increased. This indicated an influx of amino acids into the blood from the guinea-pig tissues, or hydrolysis of plasma proteins during the course of the experiment, or both. As already mentioned, a gross increase of amino acids was observed during the last 3 hr., in guinea pigs dying of anthrax. The greatest increases in concentrations were those of phenylalanine, tyrosine, histidine, lysine, alanine and methionine, and it therefore seems probable that these amino acids were not being used appreciably by the pathogen *in vivo*. Support for this fact comes from a consideration of the changes in concentration of these amino acids brought about by parallel *in vitro* growth of *B. anthracis* (see Table 2). Here, with the possible exception of methionine, the growth of the pathogen failed to decrease the concentration of these metabolites.

Table 1. *Changes in concentration of amino acids in the plasma of infected guinea pigs, produced by growth of* Bacillus anthracis

In all experiments the initial and final bacterial counts were within the ranges 1·1–7·5 × 10^6 and 0·8–1·2 × 10^9 cells/ml. of plasma, respectively.

	Conc. (µg./ml. plasma)		Average change in conc. as % of original conc.	
Amino acid	Initially	After growth of *B. anthracis*	Loss	Increase
Glutamine (plus glutamic acid)	27·3	19·7	40	—
	26·4	5·2		
	30·6	25·8		
Glutamine	Paper chromatography (3 expts.)		> 50	—
Glycine	32·9	20·9	27	—
	26·6	22·5		
Threonine	9·8	6·0	27	—
	9·6	6·4		
	7·8	7·5		
Tryptophan	7·1	6·0	15	—
	8·3	7·1		
Serine	15·8	15·8	Same	
	15·0	15·0		
Proline	17·2	19·6	—	14
	18·3	21·0		
Valine	21·3	26·4	—	22
	28·8	34·9		
Glutamic acid	Paper chromatography (3 expts.)		—	Approx. 25
Leucine + Isoleucine	Paper chromatography (4 expts.)		Same to	25
Cysteine	4·7	6·9	—	42
	6·3	8·7		
Methionine	4·2	6·3	—	46
	3·6	6·9		
	3·9	3·9		
Alanine	Paper chromatography (4 expts.)		—	25–> 50
Phenylalanine	9·8	14·7	—	55
	12·2	19·6		
Histidine	8·5	13·9	—	73
	8·3	15·0		
Tyrosine	8·8	12·8	—	85
	7·8	18·0		
Lysine	9·0	24·6	—	145
	9·6	19·5		

Table 2. *Changes in concentration of amino acids in plasma withdrawn from infected guinea pigs and incubated* in vitro

In all experiments the initial and final bacterial counts were within the ranges 2·5–7·5 × 10^6 and 1·1–1·3 × 10^9 cells/ml. plasma, respectively.

	Conc. (µg./ml. plasma)			Average change in conc. as % available amino acid	
Amino acid	Initial (*a*)	Incubated in absence of bacteria (*b*)	After growth of *B. anthracis* (*c*)	(*a*−*c*)	(*b*−*c*)
Glutamine (plus glutamic acid)	16·6	13·6	4·2	−31	−35
	29·3	35·3	27·6		
Glutamine	Paper chromatography (5 expts.)			−(25–50)	−(0–25)
Glycine	26·9	33·3	22·5	−38	−45
	30·4	31·8	14·1		
	28·4	32·1	16·9		
Threonine	9·0	11·3	4·5	−37	−53
	8·6	12·4	6·6		
Tryptophan	13·8	16·4	7·8	−48	−56
	10·1	11·3	2·4		
	7·9	10·4	6·4		
Serine	8·3	21·8	10·8	+9	−39
	12·8	19·2	15·0		
	12·3	18·9	10·5		
Proline	13·9	25·8	9·7	−47	−61
	21·0	21·3	8·7		
Valine	22·5	25·8	10·2	−73	−75
	27·0	27·9	8·9		
	23·3	25·8	0·4		
Glutamic acid	Paper chromatography (4 expts.)			−25	−25
Leucine + Isoleucine	Paper chromatography (6 expts.)			− > 50	− > 50
Cysteine	6·4	7·5	5·1	−10	−24
	6·9	8·3	6·9		
Methionine	4·3	5·1	4·3	+10	−20
	4·8	5·4	4·8		
	3·2	6·8	3·3		
	4·5	5·7	6·0		
Alanine	Paper chromatography (6 expts.)			Nil	Nil
Phenylalanine	7·5	10·5	10·5	+41	+20
	14·9	16·9	15·2		
	18·7	21·1	30·7		
	13·4	15·3	20·4		
Tyrosine	7·8	10·0	7·3	+ 8	− 3
	8·8	10·6	11·5		
	12·2	11·3	12·2		
Histidine	10·6	10·0	12·0	+26	+27
	19·2	14·6	15·8		
	15·4	16·7	23·9		
	14·1	17·4	23·1		
Lysine	12·6	15·0	14·3	+32	+1
	10·8	15·6	16·5		

Serine, glutamic acid, and cysteine occupy an intermediate position in both tables, and it is probable therefore that they are used to some extent by *Bacillus anthracis* growing *in vivo*. Proline, valine, leucine + isoleucine appear to be more extensively utilized *in vitro* than *in vivo*.

DISCUSSION

It might be expected that *Bacillus anthracis*, like other Gram-positive organisms, would take up amino acids from its environment during growth. Whether one could detect such uptake, in a complex system such as the 'free' amino acid pool of the blood of animals during the terminal bacteraemic phases of anthrax, would largely depend on the balance between the rate of uptake of individual amino acids and the efficiency of replacement by compensatory host mechanisms. However, the results presented show that a pattern of changes can be observed in the plasma amino acids of infected guinea pigs during the course of the bacteraemia. In view of the results obtained during the parallel growth *in vitro* it seems reasonable to ascribe these changes to the uptake of metabolites by the invading pathogen. It seems that some amino acids (glutamine, threonine, tryptophan, glycine) are utilized more than others.

It is difficult to relate these results to published work on the *in vitro* metabolism of *Bacillus anthracis* except to point out that Thorne (1956) showed glutamine to lie on the pathway of polyglutamic acid synthesis in *B. subtilis* and in strains of *B. anthracis*. It therefore seems likely that the decrease in concentration of this metabolite observed *in vivo* may be correlated with the synthesis of capsular polyglutamic acid. The results give no indication of the amino acids which are essential for growth and for the synthesis of virulence-determining factors. The next paper describes the use of antimetabolites in an effort to make a more specific investigation into the requirements for amino acids and other metabolites in these processes.

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REFERENCES

AGREN, G. (1950). A micromethod for the microbiological determination of amino acids. *Ark. Kemi*, **1**, 179.

CONSDEN, R., GORDON, A. H. & MARTIN, A. J. P. (1947). The identification of lower peptides in complex mixtures. *Biochem. J.* **41**, 590.

DUNN, M. S., CAMIEN, M. N., ROCKLAND, L. B., SHANKMAN, S. & GOLDBERG, S. C. (1944). Amino acids, peptides and proteins. XVII. Determination of glutamic acid in protein hydrolysates by a microbiological method. *J. biol. Chem.* **155**, 591.

FUJITA, A. & IWATAKE, D. (1931). Determination of true blood sugar without yeast. *Biochem. Z.* **242**, 43.

HAGEDORN, H. C. & JENSEN, B. N. (1923). The microdetermination of blood sugar by means of ferricyanide. *Biochem. Z.* **135**, 46.

HENDERSON, L. M. & SNELL, E. E. (1948). A uniform medium for determination of amino acids with various microorganisms. *J. biol. Chem.* **172**, 15.

HIER, S. W. & BERGEIM, O. (1945). Microbiological determination of free leucine, isoleucine, valine and threonine in dog plasma. *J. biol. Chem.* **161**. 717.

Keppie, J., Smith, H. & Harris-Smith, P. W. (1955). The chemical basis of the virulence of *Bacillus anthracis*. III. The role of the terminal bacteraemia in death of guinea pigs from anthrax. *Brit. J. exp. Path.* **36**, 315.

Smith, H. & Keppie, J. (1955). In *Mechanisms of Microbial Pathogenicity. Symp. Soc. gen. Microbiol.* **5**, 126.

Steele, B. F., Sauberlich, H. E., Reynolds, M. S. & Baumann, C. A. (1949). Media for *Leuconostoc mesenteroides* P. 60 and *Leuconostoc citrovorum* 8081. *J. biol. Chem.* **177**, 533.

Thorne, C. B. (1956). In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 68.

Troll, W. & Lindsley, J. (1955). A photometric method for the determination of proline. *J. biol. Chem.* **215**, 655.

Williams, R. J. & Kirby, H. (1948). Paper chromatography using capillary ascent. *Science*, **107**, 481.

Woiwod, A. J. (1949). A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography. *Biochem. J.* **45**, 412.

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Tempest, D. W. & Smith, H. (1957). *J. gen. Microbiol.* **17**, 739–749

The Effect of Metabolite Analogues on Growth of *Bacillus anthracis* in the Guinea pig and on the Formation of Virulence-determining Factors

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SUMMARY: Several metabolite analogues have been tested for their effect on the growth of *Bacillus anthracis* and its ability to synthesize toxin and capsular material during the terminal bacteraemic stage of anthrax in the guinea pig. 8-Azaguanine, 8-azaxanthine and to a lesser degree, ethionine, α-amino-n-butyric acid and p-fluorophenylalanine inhibited the *in vivo* growth of *B. anthracis*, whilst 2-thiouracil and pyridine 3-sulphonic acid selectively inhibited toxin production. Inhibition of capsule formation was at no time convincingly demonstrated. The annulment of these inhibitions by mixture of the analogues with appropriate metabolites gives evidence that hypoxanthine, adenine, methionine, alanine, phenylalanine and tryptophan are involved in growth *in vivo* and pyrimidines and nicotinamide in toxin synthesis.

To multiply in the tissues of a host and to produce disease a pathogen must form products in addition to new cell material: it must elaborate aggressins to combat the defence mechanisms of the host, and form toxic products or processes responsible for the symptoms of the disease. When growing in the guinea pig, *Bacillus anthracis* forms at least two aggressins, capsular polyglutamic acid and a specific oedema-producing toxin which later in the course of the disease acts to kill the host (Zwartouw & Smith, 1956; Smith, Zwartouw & Harris-Smith, 1956; Smith, Keppie & Stanley, 1955; Smith, Tempest, Stanley, Gallop & Harris-Smith, 1956). As part of an attempt to study the metabolism of *B. anthracis* when multiplying in an infected animal and producing these virulence factors, the utilization of amino acids by *B. anthracis* when growing in the blood stream of a guinea pig was studied as described in the previous paper (Smith & Tempest, 1957). These results were measurements of amino acid uptake and gave no indication about which amino acids were essential for the growth of the parasite and production of virulence factors. The present paper describes experiments to investigate the more specific role of various amino acids in this respect and the studies have been extended to include purines, pyrimidines and vitamins.

In experiments *in vitro*, the importance of each metabolite could be assessed by altering the composition of the environment so as to render it deficient in that compound. It was impossible to carry out this experimental arrangement when dealing with the metabolism of *Bacillus anthracis* in the tissues of a guinea pig. We therefore attempted to block the utilization of individual metabolites by injecting metabolite analogues into guinea pigs and to study the subsequent effects upon the growth of *B. anthracis* and on the synthesis of virulence-determining factors.

In the 10 hr. before death from anthrax in the guinea pig, capsulated *Bacillus anthracis* organisms proliferate in the blood stream and produce toxin which accumulates in the plasma. It has been pointed out (Keppie, Smith & Harris-Smith, 1955; Smith & Tempest, 1957) that in this final phase of the disease the growth rate of *B. anthracis* in the blood is remarkably constant, and it will be shown that this also applies to the rate of accumulation of free toxin. We had, therefore, a system suitable to study the effect of metabolite analogues on growth, capsulation and toxin production by *B. anthracis*. It was during this final 10 hr. of the disease that all experiments were carried out. Relatively large quantities of the analogues were injected directly into the blood stream and observations were made over relatively short time intervals. In this way, possible effects of the analogues other than a direct effect on the metabolism of the parasite were minimized. When the injection of any metabolite analogue produced a noticeable effect on the growth of *B. anthracis* or on the synthesis of virulence factors, a search was made for the metabolite which would annul this effect. Until recently, when the anthrax toxin was produced *in vitro* (Harris-Smith, Smith & Keppie, 1957) and the major part of this work completed, experiments *in vivo* were essential for metabolic studies on the synthesis of the anthrax toxin.

METHODS

*Samples of metabolites and metabolite analogues. Allo*isoleucine, α-amino-*iso*-pentane-sulphonic acid, α-amino-*iso*butane-sulphonic acid, methionine sulphoxide, S-methyl cysteine, α-methylserine, N-ethylglycine, amino-methane sulphonic acid, α-methyl glutamic acid, aspartyl-hydroxylamine, glutamyl hydrazine, and canavanine were kindly prepared by our colleague Mr S. Lovatt, to whom we are indebted. The remainder of the metabolites and their analogues were commercial products supplied by L. Light and Co., British Drug Houses Ltd., and Nutritional Biochemical Co. Most of the analogues were examined for purity either by paper chromatography (amino acid analogues) or by ionophoresis (purine and pyrimidine analogues) as described below, and were found to be free from obvious impurities.

All samples of amino acids used for annulment of inhibition experiments were free from ninhydrin-reacting impurities when examined by paper chromatography in butanol + acetic acid + water (125:30:125). Samples of purines and pyrimidines used for annulment experiments were free from ultraviolet-absorbing impurities when examined after ionophoresis on paper in butyrate buffer at pH 3·2 as described by Wade & Morgan (1954). Xanthine was the only exception; it contained small amounts of guanine, but no adenine or hypoxanthine. A sample of xanthine purified by using Dowex 50 at pH 3 was used in some of these experiments.

Selection of animals for injection. Guinea pigs (650 ± 50 g.) were infected with *Bacillus anthracis*, strain N.P. On the third day, the terminal bacteraemia was detected and followed by using stained blood films from the ears as described by Keppie *et al.* (1955). Animals having bacterial counts of

$0{\cdot}5$–5×10^6 chains/ml. blood were selected for experiment. In the normal course such animals would die 7·5–11 hr. later (Keppie *et al.* 1955).

Dosage of metabolite analogues. A single large dose (250–1000 mg.) of metabolite analogue was injected. Sometimes the dose was limited by the availability of the analogue or by an immediate toxic effect on the host. Any delayed toxic effect, due to the large dose of an analogue acting alone or in combination with the later stages of the anthrax syndrome, did not interfere with the experiment as long as there was time to take samples for observations before the guinea pig died.

Injection of analogue. When soluble in saline, the metabolite analogue was dissolved in 20 ml. and the pH value adjusted to $7{\cdot}5 \pm 0{\cdot}5$. Half this solution was injected intravenously into an exposed vein of the hind leg and the remainder intraperitoneally. When the metabolite analogue was only partially soluble in saline, it was suspended in 20 ml., warmed to 60°, adjusted to pH $7{\cdot}5 \pm 0{\cdot}5$ and allowed to cool. Ten ml. of the saturated solution were removed, filtered through Whatman no. 1 paper and injected intravenously. A suspension of the undissolved solid in the remaining solution was injected intraperitoneally.

Determination of the increase in bacterial content of the blood. At various time intervals blood films from the ear were prepared in the manner described by Keppie *et al.* (1955). The average number of chains/field was related to the number of chains/ml. blood (see Keppie *et al.* 1955) and was subject to a maximum error of $\pm 50\,\%$. In all but a few cases, when the metabolite analogue was injected the average chain length remained approximately the same as in the normal bacteraemia. In those cases when the chain length varied more than twofold from the normal average, appropriate correction was made in the recorded bacterial count.

Collection and assay of toxin. Blood (2 ml.) was obtained by cardiac puncture and mixed with ice-cold distilled water (6 ml.). The lysed blood was centrifuged (1500 *g* for 30 min) at 4° and the supernatant fluid filtered free of bacteria through Millipore filters. Twofold serial dilutions (0·2 ml.) were injected intradermally into a depilated guinea pig. After 20 hr. the highest dilution (relating to the original volume of blood) which just produced a detectable oedematous reaction was noted; the strength of the toxin was quoted in units as the reciprocal of this dilution. The assay detected twofold differences in toxin. When intravenous administration of a metabolite analogue apparently decreased toxin production, any possibility that the analogue interfered with the assay was excluded by testing a mixture of normal toxin + analogue.

Observation of bacterial capsules. Capsules were evident on the organisms in blood smears stained by methylene blue (Macfadyean reaction). When the capsule appeared diminished or absent, observations were made on wet films containing nigrosine or Indian ink.

RESULTS

Normal rate of bacterial increase and free toxin appearance in the blood during the terminal bacteraemia

Since we were interested in assessing the changes brought about in the blood of guinea pigs infected with *Bacillus anthracis* by the injection of metabolite analogues, it was important to establish that the normal degrees of bacterial increase and free toxin appearance in the blood were reasonably constant from animal to animal and not unduly affected by the other tissues of the body.

The rate of bacterial increase. Keppie *et al.* (1955) showed that the count of bacterial chains in the blood increased regularly throughout the 10 hr. which preceded death from anthrax and that this increase was remarkably constant from animal to animal. Any change in this regular pattern brought about by injection of a metabolite analogue might reasonably be ascribed to the direct effect of the analogue on the growth of the infecting bacteria since the majority of the organisms are located in the blood during this late stage of the disease (see Table 2 of Keppie *et al.* 1955).

The rate of accumulation of toxin in the blood. Samples (23) of blood taken from a number (14) of guinea pigs at different stages of the final bacteraemia were assayed for toxin. A plot of the toxin content against the number of chains of bacilli/ml. blood showed that the toxin content rose regularly with the bacterial count and was reasonably constant from animal to animal (see Figs. 5 and 6).

The amount of toxin detected here is that produced by the infecting bacteria over and above the amount which is adsorbed by the host's tissues or destroyed (or toxoided) by the growing bacteria. There is evidence which suggests that the amount of undetected toxin was considerable. Thus (i) the amount of circulating toxin *per organism* decreased as the bacteraemia progressed; this is indicated by the low slope of the normal toxin/bacterial count plot; (ii) following intravenous injection of toxin into a non-infected guinea pig the amount of circulating toxin decreased fourfold every hr.; (iii) when an amount of antitoxin capable of neutralizing more than 10 times the total amount of free toxin normally detectable at the end of the bacteraemia was injected early in the bacteraemia, this did not prevent unneutralized toxin from being found at the somewhat delayed death of the animal. Nevertheless, the rate of free toxin accumulation in the blood reflects that produced by the organism and any significant change in overall production will reflect itself on the amount of free toxin.

Preliminary survey of metabolite analogues

Fifty-seven metabolite analogues were screened for their effect on the growth, toxin production and capsulation of *Bacillus anthracis*. After injection of the analogue, blood films were examined at intervals to determine the extent of capsulation and the rate of bacterial increase but the toxin content of the blood was determined only at the end of the bacteraemia. The results are given in Table 1. Only a few of the analogues produced significant differences from the normal observations; the behaviour of these compounds was investigated in some detail.

Table 1. *The effect of metabolite analogues on growth of* Bacillus anthracis *in the guinea pig and on the synthesis of virulence-determining factors*

Metabolite analogues	Dose (mg.)	Metabolite analogues	Dose (mg.)
Metabolite analogues which were ineffective *in vivo*†			
Norleucine	1000	N-Ethylglycine	1000
Norvaline	1000	Amino methane sulphonic acid	250*
D-Norvaline	1000	α-Methyl glutamic acid	1000
Allo isoleucine	1000	β-Hydroxy glutamic acid	500
α-Amino-*iso*pentane sulphonic acid	250*	Ethyl γ-*l*-glutamate	1000
α-Amino-*iso*butane sulphonic acid	500	Aspartyl hydroxylamine	100
Isovaline	1000	Canavanine	500
Methioninol	250*	γ-Glutamyl hydrazine	150*
Methionine sulphoxide	1000	Methionine sulphone	2000
S-Methyl cysteine	1000	8-Aza-adenine	250
D-Tryptophan	500	2:6-Diaminopurine	500
Acetyltryptophan	1000	6-Mercaptopurine	500
Monochloro-*l*-tyrosine	1000	2-Thio-6-oxypurine	500
3-Amino tyrosine	500	8-Chloroxanthine	500
p-Chlorophenylalanine	500	Benzimidazole	300
o-Fluorophenylalanine	500	6-Nitrobenzimidazole	500
β-2-Thienylalanine	500	Thiothymine	500
D-Threonine	750	Isocytosine	500
Allo threonine	1000	*p*-Aminosalicyclic acid	500
α-Methylserine	500	*p*-Aminoacetophenone	250
Serinol	500	6-Chloronicotinamide	500
α-Amino-*iso*butyric acid	1000	Isonicotinyl hydrazide	100*
γ-Amino-butyric acid	1000	D-Pantothenylalcohol	1000
		2-Amino thiazole	100*
Metabolite analogues which decrease growth *in vivo*			
8-Azaguanine	250	Ethionine	500
8-Azaxanthine	250	*p*-Fluorophenylalanine	500
		α-Amino-*n*-butyric acid	1000
Metabolite analogues which decrease toxin synthesis *in vivo*			
Pyridine 3-sulphonic acid	1000	2-Thiouracil	500
3-Acetylpyridine	500*	4-Methyl 2-thiouracil	500
		2-Thio-6-amino uracil	500
		5-Bromo-uracil	500

* Toxic to guineapigs at higher concentrations.

† Injection of these analogues did not produce a rate of increase of bacterial chains in the blood significantly different from the normal (Fig. 1). The toxin amounts present at the end of the bacteraemia were not less than 64 and not more than 256 units (see p. 741). There was no significant diminution in capsule.

Analogues which decreased the in vivo *growth rate of* Bacillus anthracis

8-*Azaguanine.* This was the most effective growth inhibitor examined *in vivo.* Injection of 250 mg. brought about complete bacteriostasis for a considerable time and 50 mg. drastically decreased the rate of multiplication

(Fig. 1). Simultaneous injection of hypoxanthine annulled the effect of azaguanine; guanine was much less effective in this respect. When quantities of guanine equimolecular to that of the analogue were injected only a small decrease in the inhibition took place; this was increased by raising the proportion of metabolite to analogue. In these annulment experiments adenine replaced hypoxanthine, and xanthine behaved like guanine.

8-*Azaxanthine*. Experiments showed that the effect was similar to that described for azaguanine.

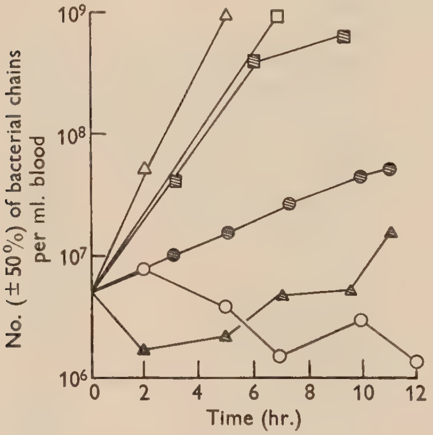

Fig. 1

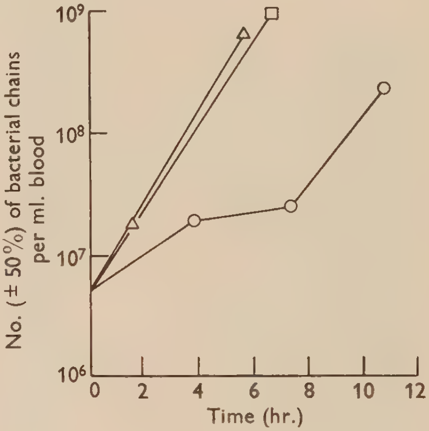

Fig. 2

Fig. 1. The inhibition of *in vivo* growth of *Bacillus anthracis* by 8-azaguanine and the reversal of this effect by purines. □, Normal untreated animal; ○, 8-azaguanine (250 mg.); △, 8-azaguanine (250 mg.)+hypoxanthine (250 mg.); ▲, 8-azaguanine (250 mg.)+guanine (250 mg.); ◍, 8-azaguanine (50 mg.); ▨, 8-azaguanine (50 mg.) +guanine (1000 mg.). The graphs shown represent the data of the most representative experiment of two to six similar experiments.

Fig. 2. The inhibition of *in vivo* growth of *Bacillus anthracis* by ethionine and its annulment by mixture with methionine. □, Normal untreated animal; ○, ethionine (500 mg.); △, ethionine (500 mg.)+methionine (500 mg.). The graphs shown represent the data of the most representative experiment of two to five similar experiments.

Ethionine. The results given in Fig. 2 show that injection of ethionine (500 mg.) decreased the growth rate, and that this effect was annulled by simultaneous injection of methionine. Leucine, isoleucine, valine, tryptophan, tyrosine, phenylalanine, glutamic acid, aspartic acid, proline, alanine, glycine, serine, arginine, lysine, threonine, cysteine and histidine, given in groups of two or three compounds, did not annul the effect.

α-Amino n-*butyric acid*. Injection of α-amino *n*-butyric acid (1000 mg.) decreased the growth rate, and this effect was annulled by simultaneous injection of alanine (Fig. 3). Injection of leucine, isoleucine, valine, tryptophan, tyrosine, phenylalanine, glutamic acid, aspartic acid, proline, glycine, threonine, serine, arginine, lysine, methionine, cysteine or histidine did not annul the effect.

p-Fluorophenylalanine. The inhibitory effect of this analogue (500 mg.) was not annulled by simultaneous injection of leucine, isoleucine, valine, glutamic acid, aspartic acid, proline, methionine, histidine, cysteine, alanine, glycine, serine, threonine, arginine, lysine or tyrosine. Phenylalanine and tryptophan partially annulled the effect but only produced complete annulment when injected together (Fig. 4).

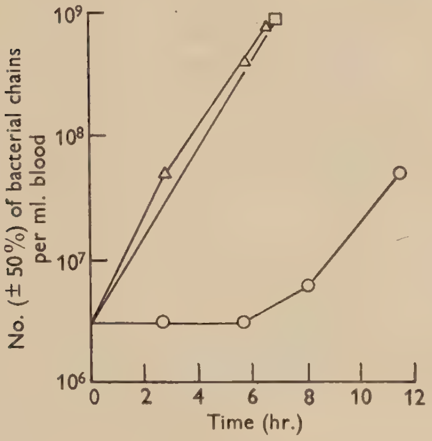

Fig. 3

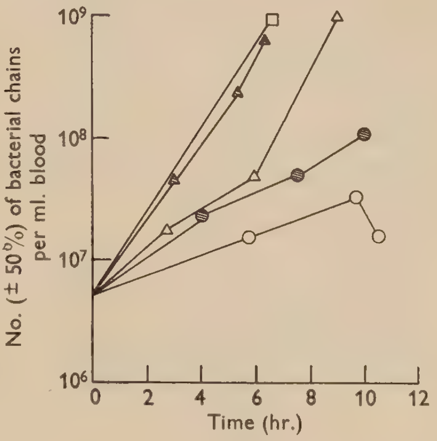

Fig. 4

Fig. 3. The inhibition of *in vivo* growth of *Bacillus anthracis* by α-amino-n-butyric acid and its annulment by mixture with alanine. □, Normal untreated animal; ○, α-amino-n-butyric acid (1000 mg.); △, α-amino-n-butyric acid (1000 mg.) + alanine (1000 mg.); The graphs shown represent the data of the most representative experiment of two to five similar experiments.

Fig. 4. The inhibition of *in vivo* growth of *Bacillus anthracis* by p-fluorophenylalanine and its annulment by mixture with phenylalanine + tryptophan. □, Normal untreated animal; ○, p-fluorophenylalanine (500 mg.); △, p-fluorophenylalanine (500 mg.) + phenylalanine (500 mg.); ,⊘ p-fluorophenylalanine (500 mg.) + tryptophan (500 mg.); ▲, p-fluorophenylalanine (500 mg.) + phenylalanine (250 mg.) + tryptophan (250 mg.). The graphs shown represent the data of the most representative experiment of two to five similar experiments.

In all these cases where the growth rate was decreased by injection of a metabolite analogue the total toxin production was also decreased. In the case of 8-azaguanine the amount of free toxin in the blood corresponded with the number of bacterial chains/ml. according to the normal toxin/growth relationship shown in Fig. 5 throughout the whole course of the inhibition. In some cases, however, there was evidence that the rate of toxin production/organism was not decreased to the same degree as the rate of growth. This difference was not apparent in the immediate period following injection of the metabolite analogue but only became evident in the later samples taken at the death of the animal. Thus, injection of ethionine, p-fluorophenylalanine and α-amino-n-butyric acid into different animals produced the following increased toxin content of the blood at the indicated bacterial count (chains/ml.); the normal toxin content in units (see page 741) which corresponds to the bacterial count in the uninhibited system is given in brackets: ethionine, 48 (12) at $4\cdot5\times10^{7}$, 96 (16) at $7\cdot5\times10^{7}$, 64 (24) at $9\cdot0\times10^{7}$; *p*-fluorophenylalanine 32 (12) at $4\cdot5\times10^{7}$, 48 (24) at $9\cdot0\times10^{7}$, 64 (32) at $1\cdot5\times10^{8}$, *α*-amino-*n*-butyric acid 48 (12) at $4\cdot5\times10^{7}$, 128 (16) at $7\cdot5\times10^{7}$, 96 (48) at $3\cdot9\times10^{8}$. It seems therefore that growth and toxin production can be divorced from one another; this is amply confirmed by the results shown in the next section.

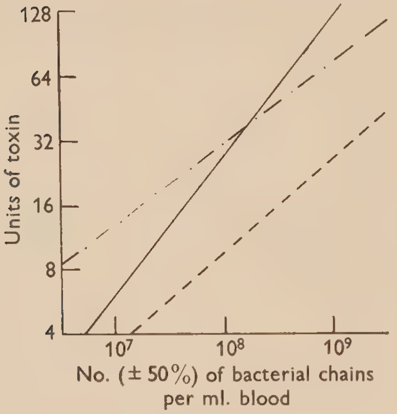

Fig. 5

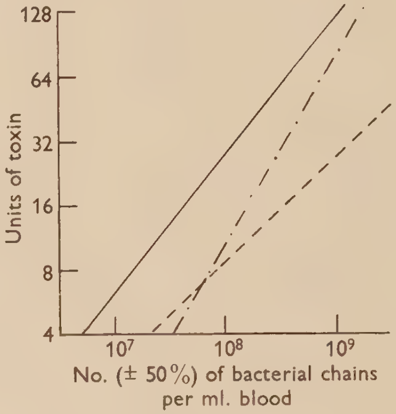

Fig. 6

Fig. 5. The inhibition of toxin production by 2-thiouracil and its annulment by uracil. ——, Growth/toxin curve in untreated animals. $y=0\cdot62x-0\cdot46\pm0\cdot27$ (fiducial limits for $P=95\,\%$). – – –, Growth/toxin curve in animals treated with 500 mg. 2-thiouracil. $y=0\cdot43x-2\cdot45\pm0\cdot46$ (fiducial limits for $P=95\,\%$). —·—·—, Growth/toxin curve in animals treated with 2-thiouracil (500 mg.) + uracil (500 mg.) $y=0\cdot37x-1\cdot49\pm0\cdot48$ (fiducial limits for $P=95\,\%$).

Fig. 6. The inhibition of toxin production by pyridine 3-sulphonic acid and the effect of simultaneous injection of nicotinamide. ——, Growth/toxin curve in untreated animals. $y=0\cdot62x-0\cdot46\pm0\cdot27$ (fiducial limits for $P=95\,\%$). – – –, Growth/toxin curve in animals treated with pyridine 3-sulphonic acid. $y=0\cdot48x-2\cdot88\pm0\cdot45$ (fiducial limits for $P=95\,\%$). —·—·—, Growth/toxin curve in animals treated with pyridine 3-sulphonic acid (1000 mg.) + nicotinamide (500 mg.). $y=0\cdot92x-6\cdot42\pm0\cdot47$ (fiducial limits for $P=95\,\%$). N.B. Admixture with nicotinamide (1000 mg.) was too toxic to the guinea pig for observation to be made.

Metabolite analogues which decrease the rate of toxin production without materially decreasing growth rate or capsule production

2-*Thiouracil.* Samples (24) of blood from animals (18) which had been injected with thiouracil (500 mg.) were taken at different stages in the terminal bacteraemia. The mean rate of increase of bacteria (log number of chains/ml. blood/hr.) in these thiouracil-treated animals 0·33 (S.E. 0·02), was not significantly different from the normal rate 0·34 (S.E. 0·01). However, the curve of the toxin content of the blood plotted against number of chains/ml. was significantly different from that of the normal curve (Fig. 5). Toxin production had been inhibited although the rate of growth of the *Bacillus anthracis* was normal. Simultaneous injection of uracil (500 mg.) with thiouracil (500 mg.) increased toxin production (see Fig. 5) to normal values although the slope of toxin increase with bacterial count was somewhat lower than the normal slope. In smaller scale experiments cytosine, thymine or orotic acid annulled the effect of 2-thiouracil.

4-*Methyl-2-thiouracil, 2-thio-6-amino-uracil and 5-bromo-uracil* were not examined further than in the preliminary tests since it seemed likely that their mode of action would have been the same as 2-thiouracil.

Pyridine 3-sulphonic acid. Pyridine 3-sulphonic acid (1000 mg.) was injected into a number of animals (10) and samples (15) of blood taken for analysis as described for the experiments with thiouracil. Again, the rate of increase of bacteria (log number of chains in the blood/hr.) in the treated animals (0·32 s.e. 0·02) was not significantly different from the normal rate but toxin formation was inhibited (Fig. 6). Simultaneous injection of nicotinamide (500 mg.) partially annulled the effect of pyridine 3-sulphonic acid in the later stages of the bacteraemia (see Fig. 6), but simultaneous injection of uracil did not.

3-*Acetylpyridine.* This metabolite analogue was rapidly toxic to test animals at doses in excess of 500 mg. At this dose, a small but significant decrease in amount of free toxin/organism occurred, but since the rate of growth of the organism was much stimulated by the injection of this metabolite analogue (this corrects the statement of Tempest & Smith, 1956), the results are difficult to assess.

The analogues which affected toxin production did not significantly affect the capsulation of the organisms.

DISCUSSION

Some success has attended the use of metabolite analogues to investigate the amino acids and other nutrilites important in the *in vivo* growth and toxin production of *Bacillus anthracis.* There were difficulties attending these experiments, thus, the trapping of growing organisms in the reticulo-endothelial system, the growth of *B. anthracis* in chains rather than single organisms, the possible production of toxin by organisms in sites other than the blood, the unknown rates of adsorption and detoxification of toxin by the tissues of an infected guinea pig and, above all, the effect of the metabolite analogue on all these factors. This allowed only a semi-quantitative treatment of results. However, it was shown that some metabolite analogues did decrease growth *in vivo* and concurrently the toxin production of *B. anthracis*, while others suppressed toxin production without interfering with growth. The annulment of these effects by corresponding metabolites serves as evidence for the specific use of these metabolites by *B. anthracis* in the appropriate metabolic processes *in vivo.*

8-Azaguanine was the most effective growth inhibitor. The specificity of the annulment of this inhibition is somewhat at variance with the results of workers who used other organisms (Creaser, 1956) especially in that guanine is less able to counteract the effect of 8-azaguanine with *Bacillus anthracis* in guinea pigs, than is adenine and hypoxanthine. It is possible that the behaviour of guanine and of xanthine is due to their low solubility in the tissue fluids.

It appears from the annulment of the effects of ethionine, *p*-fluorophenylalanine and α-amino-*n*-butyric acid that the unimpaired metabolism of methionine, phenylalanine, alanine and tryptophan are essential for *in vivo* growth of *Bacillus anthracis.* All these amino acids are present in adequate concentrations in the free amino acid pool of the guinea pig plasma but only tryptophan is taken up by *B. anthracis* in appreciable quantities (Smith & Tempest, 1957).

The inhibition of toxin synthesis by 2-thiouracil or pyridine 3-sulphonic acid and the annulment of their effects by various pyrimidines or nicotinamide, respectively, suggests that the latter compounds are involved in the processes leading to toxin production. It is possible that the two metabolite analogues inhibit the production of only one of the two factors in the anthrax toxin (Smith *et al.* 1956) but this point has not been investigated.

Only one of the metabolite analogues examined, namely methionine sulphone, produced a noticeable effect on capsulation. This was apparent as a loss in staining property but examination of the organisms in wet Indian ink preparations showed the general structure of the capsule to be intact. It is of interest that Thorne (1956) showed that alanine and phenylalanine (analogues of which limit growth *in vivo*) are intimately connected with capsule synthesis in closely related organisms.

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REFERENCES

Creaser, E. H. (1956). The assimilation of amino acids by bacteria. 22. The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus. Biochem. J.* **64**, 539.

Harris-Smith, P. W., Smith, H. & Keppie, J. (1957). Production *in vitro* of the anthrax toxin previously recognized *in vivo. J. gen. Microbiol.* **16**, viii.

Keppie, J., Smith, H. & Harris-Smith, P. W. (1955). The chemical basis of the virulence of *Bacillus anthracis.* III. The role of the terminal bacteraemia in death of animals from anthrax. *Brit. J. exp. Path.* **36**, 315.

Smith, H., Keppie, J. & Stanley, J. L. (1955). The chemical basis of the virulence of *Bacillus anthracis.* V. The specific toxin produced by *B. anthracis in vivo. Brit. J. exp. Path.* **36**, 460.

Smith, H. & Tempest, D. W. (1957). The uptake of amino acids during the terminal bacteraemia in guinea pigs infected with *Bacillus anthracis. J. gen. Microbiol.* **17**, 731.

Smith, H., Tempest, D. W., Stanley, J. L., Gallop, R. C. & Harris-Smith, P. W. (1956). The chemical basis of the virulence of *Bacillus anthracis.* VII. Two components of the anthrax toxin: their relationship to known immunising aggressins. *Brit. J. exp. Path.* **37**, 263.

Smith, H., Zwartouw, H. T. & Harris-Smith, P. W. (1956). The chemical basis of the virulence of *Bacillus anthracis.* VIII. Fractionation of the intracellular material of *Bacillus anthracis. Brit. J. exp. Path.* **37**, 361.

TEMPEST, D. W. & SMITH, H. (1956). The effect of metabolite analogues on some virulence factors of *Bacillus anthracis* in the guinea pig. *J. gen. Microbiol.* **15**, v.
THORNE, C. G. (1956). Capsule formation and glutamyl polypeptide synthesis by *Bacillus anthracis* and *Bacillus subtilis*. In *Bacterial Anatomy. Symp. Soc. gen. Microbiol.* **6**, 68.
WADE, H. E. & MORGAN, D. M. (1954). The analysis of adenosine triphosphate and adenosine diphosphate preparations by paper ionophoresis. *Biochem. J.* **56**, 41.
ZWARTOUW, H. T. & SMITH, H. (1956). Polyglutamic acid from *Bacillus anthracis* grown *in vivo*: Structure and aggressin activity. *Biochem. J.* **63**, 437.

(*Received* 4 *July* 1957)

Laughton, N. & Davies, J. (1957). *J. gen. Microbiol.* **17**, 750–757

Animal Strains of Group G Streptococci and their Serological Typing

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SUMMARY: A brief review of group G streptococci and their typing is given with particular reference to canine strains. Two of the Australian type strains of Simmons & Keogh (1940) were found to be in the group phase and useless for comparative purposes. Four types Kennett, Maxie, Airedale and R51/755 were clearly defined in addition to the Australian types Harrison and Cuthell by reciprocal absorption tests using slide agglutination. Fifty-four other strains of which 35 were of animal and 19 of human origin were examined for type. Of the 38 strains from animal sources, 33 belonged to one of the two types Kennett or Maxie, strains of which came exclusively from animal sources. Six of the human strains and one of the animal ones were untyped.

Type R51/755 which had been found previously by Maxted (1949) to possess the M28 antigen owed its type-specificity entirely to this antigen and this was not found to be present in any of the other types.

The substances responsible for type-specificity in types Kennett, Maxie, and Airedale differed from those of types Harrison and Cuthell and like that of type R51/755 appeared to be protein in nature. The type-specific substances of types Airedale and Maxie had exactly the same characters as the M28 antigen in being sensitive to peptic but resistant to tryptic digestion. The protein antigen of type Kennett differed from the other three protein type-specific substances in showing only moderate resistance to tryptic digestion.

Streptococci of group G which share a common group-specific polysaccharide in the Lancefield serological classification were first defined by Hare & Lancefield (1935) among a collection of β-haemolytic strains mainly from human sources. Since then numerous workers have isolated strains of this group from normal human throat, nose, vagina and skin, but they have seldom been associated with serious human infection. On the other hand, there is more evidence, particularly of the circumstantial type, that some group G strains are pathogenic to animals, particularly to dogs (Hare & Fry, 1938*a*, *b*; Stableforth, 1938; Minett & Ellis, 1940; Laughton, 1948; Laughton & Larin, 1954).

In her studies of 55 strains of 'minute-colony' haemolytic streptococci of human origin, Bliss (1937) found that 5 of them belonged to group G. These were shown to possess a specific type substance which was identical with the type-specific substance which Bliss had found in her commonest type belonging to group F called Hav. She proposed the designation type I in both groups for strains containing this type-specific antigen. Simmons & Keogh (1940) undertook the serological typing of group G strains of human origin. Of the 78 strains which they examined 70 were of the 'large-colony' type and 8 of the 'minute-colony' type. The typing technique used was slide agglutination with cocci which had been subjected to tryptic digestion in order to ensure stable suspensions. The 'large-colony' strains fell into three serological types: 62 belonged to type Cole, 7 to type Auburn, and 1 to type Harrison. Four only of the 8 'minute-colony' strains belonged to a single type which was named Cuthell. These strains were not compared with 'type I' of Bliss.

Maxted (1949) showed that a group G strain J 23 possessed an antigen which was identical with the group A type M 28 protein antigen described by Lancefield (1943), in its ability to absorb the corresponding antibody and to stimulate the production of type M 28 antibody. A group C strain D 10 also contained the same antigen, but a further examination of this strain suggested that the M 28 antigen was not the major type-specific antigen of this strain.

The present authors, having a collection of group G strains from animal sources, decided to compare them with the Australian type strains from human sources to ascertain whether type could be related to source and because typing might be of use in epidemiological investigations of animal infection with group G streptococci. The strains were also examined for the presence of the M 28 antigen to ascertain whether it was of major or minor importance in determining type-specificity. Unfortunately, the original plan of comparing animal and human strains with reference to type became impossible as two of the Australian type strains, which incidentally were lodged with the National Collection of Type Cultures, Colindale, London, N.W. 9, as soon as they arrived in this country, were found to have lost their type-specificity.

The animal strains used comprised a number isolated by one of us some years previously and kept in a dried state; these were of canine origin with the exception of two strains, one of which came from a spontaneously occurring infection in a laboratory ferret and the other from the tonsils of a normal monkey. The strain Do. 178 (Kennett) which was also used by Laughton & Larin (1954) was isolated from the lungs of a puppy which died of pneumonia. The strain R 51/755 was supplied by Mr W. R. Maxted who had found it to contain the M 28 protein antigen; the strain J 23 was no longer available. The group A strains Small containing the M 28 antigen and *Streptococcus pyogenes* type 1, which was used to absorb the group A antibody from the serum of Small, were supplied by the NCTC.

METHODS

Grouping of strains. All the strains used were grouped at least twice by precipitin tests with extracts made by Lancefield's and by Fuller's methods. Some group G strains gave weak cross-reactions with the sera of other groups, particularly with groups A and L, and less frequently with groups B and C. Although the majority of cross-reactions were obtained with the acid extracts some were also seen with formamide extracts. Inter-group cross-precipitation was recorded by Bliss (1937), Maxted (1949), Sharpe (1952) and Perry & Briggs (1955). In some cases these cross-reactions may be due to the nucleoprotein antigen common to all streptococci and in others, it is due to the chance possession of the same antigen by individual strains of different Lancefield serological groups.

Typing of strains. The method of typing was by slide agglutination with crude and absorbed sera. In reading the tests a positive result was recorded only when marked and clearly visible naked-eye agglutination appeared within 60 sec. of mixing the suspension of test organism and serum, and a control test of the same suspension and saline remained stable.

Suspensions of organisms. Standard suspensions of about 30,000 million cocci/ml. were used. Trouble was experienced at first through loss of agglutinability when organisms were kept at 4° for a few days in physiological saline containing 0·2 % (v/v) formalin. After trials with varying methods of preservation such as heating, 1/10,000 thiomersalate, doubling the strength of the formalin preservative or using 0·5 % (w/v) phenol in physiological saline, the last named was found to provide suspensions which showed the least loss of agglutinability up to a period of a month. Suspensions prepared from the same strains at different times were not always equally agglutinable and therefore had to be checked periodically, and tests were repeated several times for confirmation. Stableforth (1932) mentions this variability of agglutination. A more serious difficulty was to obtain stable suspensions; this trouble was frequent and was even variable with regard to the same strain at different times, but it was invariably overcome by patience and various expedients. When everything else failed the cocci were subjected to tryptic digestion and, for the investigation of the nature of the type-specific substances, to peptic digestion as well by the methods given by Maxted (1949).

Immune sera. The cocci were grown for 24 hr. in tryptic digest broth containing 0·1 % (w/v) glucose and washed with physiological saline containing 0·4 % (v/v) formalin and resuspended in saline containing 0·2 % (v/v) formalin at about 3000 million cocci/ml. and inoculated intravenously into rabbits whose blood serum had been previously tested for the presence of normal agglutinins since some rabbits had been found to possess these in low titre. Such rabbits were not used. An initial dose of 0·5 ml. of suspension was followed by 1 ml. on the 2nd and 3rd days when no untoward reaction occurred. Subsequently, three to six 3-day courses with 4 days rest in between were given. Some rabbits were adversely affected by these antigens and the loss of several early in the work showed the need for careful regulation of dosage. Maxted (1949) reported the death of three rabbits during their immunization with the group G streptococcus strain J23. Test bleedings were made after three or four courses of immunization to ascertain the titre and quality of the antibodies by precipitin and agglutination tests. Type-specific antibodies normally showed early but when the immunization was prolonged group antibodies, as shown by positive cross-precipitin tests, became prominent. The titres of the crude sera varied from 1/800 to over 1/2000 in slide agglutination tests.

Absorption of sera. The method by which the sera was absorbed was that of Griffith (1926). After centrifugation the sera were Seitz-filtered into bijoux bottles and preserved with 1/10,000 thiomersalate.

The minimum adsorptive dose (MAD) of each strain for each homologous serum was measured by the volume of glucose broth which supported a 24 hr.

growth sufficient to absorb all the agglutinins from 0·05 ml. of serum. The volume needed for individual sera varied from 25 to 125 ml. After preliminary tests with our own strains, reciprocal absorption tests of the Australian prototype strains and some of our strains which appeared to be different, and the group A strain Small, were carried out. In absorbing the sera a good group-antibody absorbing strain was used when necessary in addition to a heterologous type strain, because organisms in the type-specific phase did not always complete the removal of the group antibody unless unreasonable amounts were used, for example, in one test 4 MAD (minimum adsorptive dose) of Cuthell failed to remove all the group antibody from an Auburn serum. After absorption each serum was tested with all the other strains. In the event of a cross reaction, as happened for example between the Kennett serum absorbed with Auburn, and Maxie suspension, or the Maxie serum absorbed with R51/755, and Airedale suspension, the cross-reacting strain was subsequently used as well as other strains to ensure type-specificity. Suitable diagnostic sera purified to contain the major type-specific antibodies only were thus made and used in the testing of the other 54 strains of group G. When an organism was identified as a particular type it was used to absorb the crude serum of the prototype strain with which it had given a positive reaction, using the corresponding MAD. In the case of a few organisms slightly more than 1 MAD was needed to absorb the prototype agglutinins, in other cases when 1 MAD was used, the prototype strain gave a negative reaction but the absorbing strain still gave a positive reaction but with a reduced titre indicating a difference of sensitivity to agglutination.

RESULTS

Table 1 is representative of the results obtained when the crude sera of 8 group G prototype strains and the group A strain Small containing the M28 protein antigen were cross tested. The extent to which cross-agglutination between strains is representative of group antibodies is speculative because, in an agglutination test in which the surface antigens are the principle participants, adequate contact of a group polysaccharide which might be deeply placed within the bacterial cell and its corresponding antibody in the serum under test, might not be effected; indeed, according to Lancefield (1941) there is no group agglutination corresponding to the group precipitin reaction and neither can variation in non-type-specific agglutination be correlated with the amount of group-specific carbohydrate antibody in the serum because in some cases a positive result may be due to the reaction of the non-specific protein antigen common to all streptococci with the corresponding antibody, but much depends on the quality of the sera used and whether the organism is in a highly specific state, or de-nuded of its more superficial antigens.

This being so, the information to be gained from Table 1 is mainly in the consideration of the negative or low titre results. These results reflect the quality of the sera with regard to type-specificity and also indicate which types are well protected from group agglutination by their type-specific

Table 1. *Slide-agglutination tests of crude streptococcal grouping sera*

Strepto-coccal strain	Group A serum Small	Group G sera							
		R 51/755	Auburn	Cole	Harrison	Cuthell	Kennett	Maxie	Airedale
Small	+++	+++	−	−	−	−	±	±	−
R 51/755	+++	+++	++	+++	++	±	+++	+	+++
Auburn	±	+++	+++	+++	+++	±	++	+	+++
Cole	±	+++	+++	+++	+++	±	+	±	+++
Harrison	−	+	±	±	+++	−	−	−	±
Cuthell	−	−	±	±	±	+++	−	−	−
Kennett	±	+++	++	++	+++	±	+++	+	+++
Maxie	±	+++	+++	+++	+++	+	+++	+++	+++
Airedale	±	−	±	−	−	−	±	±	+++

Agglutination values: + + +, 1 in 200 or more; + +, 1 in 100; +, 1 in 50; ±, less than 1 in 50; −, negative reaction.

antigens. Among the group G strains, the serum Cuthell and to a lesser extent the serum Maxie are the most type-specific and the organisms Cuthell, Harrison and Airedale appear to be the best protected by their specific antigens from the group-specific and non-specific agglutinins.

The third point of interest is the high degree of reciprocal cross reaction between the group A strain Small and the group G strain R 51/755 which is not seen in the case of the other group G strains. This finding indicates the presence of the common M 28 protein antigen.

Reciprocal absorption tests

Reciprocal absorption tests between the strains Auburn and Cole with 3 different sera against each showed these two strains to be identical because with one exception, when slightly more was needed, 1 MAD of either strain completed the removal of all the agglutinins in the 12 tests; moreover, 1 MAD of the strain Harrison or strain R 51/755, which were good group antibody absorbers, removed all the agglutinins of each of the 6 sera. It was therefore concluded that Auburn and Cole strains were in the group phase and the strain Cole was not used again in the investigation. Furthermore, the results of numerous and repeated absorption tests with strain Auburn and the 6 prototype strains established the identity of the latter as distinct types, but certain minor cross-reactions already mentioned above seemed to indicate the sharing of less significant antigens by strains Kennett and Maxie, and by strains Maxie and Airedale. The sharing of such minor antigens, however, could not be satisfactorily confirmed by repeated reciprocal absorption tests and these slight cross-reactions did not interfere with major type definition which was clear-cut in each case.

Typing of additional strains

Fifty-four other strains were investigated for type-specificity in the way described above. Table 2 shows the type distribution and host source of these strains. The 3 Cuthell strains obtained from human sources were of the 'minute-colony' type. All other strains were of the 'large-colony' type. Thirty-five strains were of animal origin and 19 of human origin. The types Kennett and Maxie came exclusively from animal sources and accounted for 31 of the 35 animal strains. Six human and 1 animal strain remained untyped.

Table 2. *Typing of 54 group G streptococcal strains from various sources*

Type ...	Cuthell	Harrison	Airedale	Kennett	Maxie	R 51/755	Untyped
No. ...	3	4	6	17	14	3	7
Source...	3 Human	3 Human 1 Canine	4 Human 2 Canine	12 Canine 2 Milk 1 Ferret 2 Animal (? origin)	13 Canine 1 Monkey	3 Human	6 Human 1 Canine

Nature of the type-specific substances

The group G strains, and the type A strain Small as a control, were subjected to tryptic and peptic digestion in order to find out something of the chemical nature of the substances determining their type specificity and their relation to the M28 protein antigen. Lancefield (1943) showed and Maxted (1949) confirmed that the M28 antigen withstands tryptic digestion for 72 hr. but is inactivated by peptic digestion. Each group G strain was digested with each of the two enzymes separately and tests were made at 24, 48 and 72 hr. The strains Auburn and Cole being in the group phase, were tested against their own crude sera and, as was to be expected, both the tryptic- and peptic-digested cocci reacted to titre on account of the group polysaccharide. The other 6 type strains and the group A strain Small were tested against their homologous sera after removal of their respective group antibodies. The strains Harrison and Cuthell also reacted to titre at 72 hr. with both treated suspensions, indicating that their type-specific substances were unlikely to be proteins. On the other hand, the strains Airedale, Maxie, Kennett, R 51/755 and Small gave negative reactions after 24 hr. digestion with pepsin. With the exception of Kennett, the 72 hr. tryptic digested suspensions of these organisms reacted to titre with their respective homologous sera. The strain Kennett repeatedly showed a sharp fall in agglutination titre after 24 hr. treatment with trypsin and it gave a negative result after 48 hr.

These findings showed that the type-specific antigens of strains Maxie and Airedale had the same characters as the M28 antigen in relation to tryptic and peptic digestion, but that that of strain Kennett differed in being only moderately resistant to tryptic digestion. To confirm that these strains did not, in fact, contain the M28 protein as well as their major type-specific antigens, the reciprocal absorption of agglutinin tests among Kennett, Maxie, Airedale, R 51/755 and Small were repeated after the removal of the group antibodies by suitable strains of groups G and A respectively. The results showed no decrease of titre in either direction among the strains Small and R 51/755, and Maxie, Kennett and Airedale, but in sharp distinction there was complete reciprocal absorption between Small and R 51/755 based on their common possession of the M28 antigen, and this antigen appeared to be the only type-specific antigen of R51/755 because the type-specific agglutinins of its serum were completely removed by Small. It also completely absorbed the type-specific antibody of the serum Small.

DISCUSSION

With regard to the nomenclature of types within group G, since the Australian type Cuthell was not compared with type 1 (Hav.) of Bliss (1937) these two strains may or may not have been identical types as apparently according to Simmons & Keogh (1940) the 'minute-colony' strains of group G comprise more than one type, our 3 such strains none of which came from animal sources, were type Cuthell.

It is also unfortunate that the Australian type strains Cole and Auburn had lost their type-specificity when received by us. We did attempt to regain this by animal inoculation in mice but without success, although a localized inflammatory reaction was obtained. Had they been in the type-specific state we may well have found that they corresponded with two of our named types. Some of our untyped strains may also have been in the group phase, in fact, one was found to be so. It seems probable that the types Kennett and Maxie are primarily animal parasites, but more strains of both animal and human origin need to be typed to confirm this probability.

Maxted (1949) thought that in the case of the group C strain D10 the M28 protein antigen it contained was not the major type-specific antigen, but it is so in the case of the group G strain R51/755. We did find slight evidence of the sharing of minor type-specific antigens among some of our types but we were unable to confirm this.

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REFERENCES

Bliss, E. A. (1937). Studies upon minute haemolytic Streptococci. *J. Bact.* **33**, 625.

Griffith, F. (1926). Types of haemolytic streptococci in Scarlet fever. *J. Hyg., Camb.* **25**, 385.

Hare, T. & Fry, R. M. (1938*a*). Preliminary observations of infection in dogs by β-haemolytic streptococci. *Vet. Rec.* **50**, 213.

Hare, T. & Fry, R. M. (1938*b*). Clinical observations on β-haemolytic infections of dogs. *Vet. Rec.* **50**, 1537.

Hare, R. & Lancefield, R. A. (1935). The serological differentiation of pathogenic and non-pathogenic strains of haemolytic streptococci from parturient women. *J. exp. Med.* **61**, 335.

Lancefield, R. A. (1941). Specific relationship of cell composition to biological activities of haemolytic streptococci. *Harvey Lect.* **36**, 261.

Lancefield, R. A. (1943). Studies on the antigenic composition of group A haemolytic streptococci. *J. exp. Med.* **78**, 465.

LAUGHTON, N. (1948). Canine β-haemolytic streptococci. *J. Path. Bact.* **60**, 471.

LAUGHTON, N. & LARIN, N. M. (1954). Interaction of the natural host and the canine hepatitis virus on the infectivity of group G streptococci. *Nature, Lond.* **173**, 84.

MAXTED, W. R. (1949). Occurrence of the 'M' substance of type 28 group A streptococci of Lancefield groups B, C and G. *J. gen. Microbiol.* **3**, 1.

MINETT, F. C. & ELLIS, A. A. B. (1940). Streptococcal infections in dogs. *Vet. J.* **96**, 438.

PERRY, K. D. & BRIGGS, C. A. E. (1955). A common type antigen in streptococci of groups D and E. *J. Path. Bact.* **70**, 546.

SHARPE, E. M. (1952). Occurrence of a common type antigen in streptococci of groups D and N. *J. gen. Microbiol.* **7**, 192.

SIMMONS, R. T. & KEOGH, E. V. (1940). The physiological characters and serological types of haemolytic streptococci of groups B, C and G from human sources. *Aust. J. exp. Biol. med. Sci.* **18**, 151.

STABLEFORTH, A. W. (1932). Studies in Bovine Mastitis. *J. comp. Path.* **45**, 185.

STABLEFORTH, A. W. (1938). Streptococcal infections of animals and their treatment. *Vet. Rec.* **50**, 1203.

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HAWKER, L. E., HEPDEN, P. M. & PERKINS, S. M. (1957). *J. gen. Microbiol.* **17**, 758–767

The Inhibitory Effect of Low Temperature on Early Stages of Zygospore Production in *Rhizopus sexualis*

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SUMMARY: Colonies of *Rhizopus sexualis* (Smith) Callen on 1 % (w/v) malt agar at 20° show the first stages in conjugation approximately 40 hr. after inoculation and the first mature zygospores after a further 32–34 hr. Transfer to 5–10° prevents the initiation of conjugation and inhibits the further development of the majority of pairings in which the gametangia are not fully delimited at the time of transfer. Fully delimited gametangia continue to develop and to produce mature zygospores at the low temperature. This effect is almost entirely independent of the composition and pH value of the medium. Sporangia are produced in greater numbers at the low temperature than at 20°. The effect of low temperature on formation of zygospores of *Mucor hiemalis*, *Zygorrhynchus macrocarpus* and *Z. moelleri* is essentially similar to that with *R. sexualis*, but *Phycomyces blakesleeanus* shows only slight inhibition at 5° and none at 7° or over. The significance of the inhibitory effect is discussed.

When cultures of *Rhizopus sexualis* (Smith) Callen (a homothallic species of the Mucoraceae) showing all stages in zygospore formation were stored at 7° most of the young zygospores continued to develop and reached maturity, although the rate of development was decreased by the low temperature. Early stages in conjugation already present on transfer to low temperature did not result in zygospores and no further conjugations took place. When the cultures were replaced at 20° conjugations again took place. It was hoped that further study might elucidate the mechanism of the particular stage in reproduction at which the inhibitory effect of low temperature was most marked.

The literature relating to the general effect of temperature on growth and reproduction in fungi has been reviewed (Hawker, 1950, 1957; Lilly & Barnett, 1951). Although there is little detailed information about the effects of temperature on zygospore formation in the Mucoraceae, the available information shows that these effects are complex. Zygospore production by *Sporodinia grandis* (= *Syzygites megalocarpa*) is favoured by higher temperatures than those optimal for sporangial production (Baker, 1931). Zygospores of *Thamnidium elegans* are formed only after exposure to temperatures of 6–7° (Hesseltine & Anderson, 1956). Robins & Schmitt (1945) showed that temperature influences zygospore formation by *Phycomyces blakesleeanus* by an indirect effect on hydrogen-ion concentration.

The type of asexual reproduction in pleomorphic members of the Mucoraceae is controlled by the interaction of a number of factors, including temperature.

A striking example is *Choanephora cucurbitarum* (Barnett & Lilly, 1950); at 25° conidia are formed more frequently than sporangia, at 30° sporangia predominate and at 31° only sporangia are formed.

METHODS

Material. The strain of *Rhizopus sexualis* used was derived from the original isolate from strawberry (Smith, 1938; Callen, 1940). Other species used (homothallic species, *Zygorrhynchus macrocarpus*, *Z. moelleri*; heterothallic species, *Mucor hiemalis*, *Phycomyces blakesleeanus*) were taken from the collection in this Department. All organisms were maintained on malt agar slopes.

Culture methods. Unless otherwise stated, the cultures were grown on 1% (w/v) malt agar, since this medium at suitable temperatures allowed the formation of a moderate number of zygospores without the development of sufficient aerial mycelium to make observations difficult. Plate cultures (in Petri dishes, 9 cm. diameter) were inoculated with disks, 7 mm. diameter, cut from the advancing edges of young colonies before conjugation had begun, thus ensuring that no young zygospores were included in the inoculum. Slide cultures, in which microscope slides were coated with a film of agar medium and supported on glass rods in damp sterile Petri dishes, were inoculated with smaller pieces of young agar cultures.

Estimation of growth and reproduction

Mycelial growth. The average diameter of colonies was measured at suitable intervals.

Zygospore counts. With plate cultures of *Rhizopus sexualis* lines at right angles through the centre of the inoculum were marked on the bottom of the plate. Arbitrarily chosen points were marked along these lines and the number and stage of development of the zygospores in microscopic fields at these points were recorded at intervals. Microscopic fields at arbitrarily determined points were also examined in slide cultures. These methods were not entirely suitable for use with the other species studied. Zygospores of *Zygorrhynchus* spp. are small and crowded and counts can be made only on slide cultures. With the plate cultures the sharply demarcated central zone containing zygospores was outlined in Indian ink on the base of the Petri dish before transfer to low temperature and any further conjugations seen beyond this outline were later recorded.

The heterothallic *Mucor hiemalis* also produces small crowded zygospores along the line of contact of the 'plus' and 'minus' colonies and aerial mycelium along this zone is profuse. It was possible to count the zygospores at various stages of development along a short arbitrarily chosen part of the zone.

Zygospores of *Phycomyces blakesleeanus* are so large that the total number on a plate could be recorded.

Definition of stages in development of zygospores

A. *Early progametangial stage*: progametangia just recognizable, slightly swollen (Fig. 1A).

B. *Progametangial stage*: progametangia much enlarged, cytoplasm denser than in supporting hyphae (Fig. 1B).

C. *Early gametangial stage*: gametangia distinguishable but not cut off by cross-walls (Fig. 1C).

D. *Gametangial stage*: gametangia delimited by cross-walls (Fig. 1D).

E. *Fusion stage*: wall between gametangia dissolved, no dark pigment (Fig. 1E).

F. *Young zygospore stage*: spore rounded-off, enlarged, brown pigment present, warty thickenings developing on wall (Fig. 1F).

G. *Mature zygospore stage*: spore further enlarged, black, well-developed warts on wall (Fig. 1G).

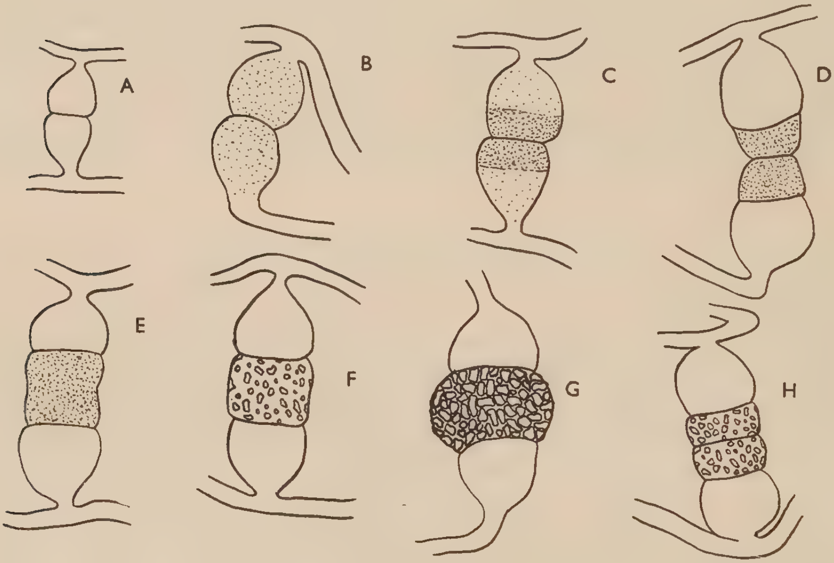

Fig. 1. Formation of zygospores of *Rhizopus sexualis*. A = early progametangial stage; B = progametangial stage; C = early gametangial stage; D = gametangial stage; E = fusion stage; F = young zygospore; G = mature zygospore; H = abnormal zygospore in which wall between gametangia has failed to dissolve (stage X in text, p. 760). A–H, ×80.

Occasionally the gametangia were delimited normally (stage D) but the wall between them did not break down. Various degrees of pigmentation and sculpturing of the gametangial wall followed, but normal zygospores were not produced. All such abnormal forms were recorded as X (Fig. 1H).

The description of the stages given above refers to *Rhizopus sexualis*, but the scheme was also valid for the other species used, due allowance being made for differences in type of wall sculpture, presence of bristles as outgrowths of the suspensor cells (in *Phycomyces blakesleeanus*), etc.

Zygospore index. In order to assess the general stage of zygospore production reached by a particular colony, an arbitrary Zygospore Index (ZI) was calculated. The stages A–G were given an arbitrary value of 1–7 respectively. The total number of pairings at any particular stage seen in the microscopic fields chosen as indicated above, was multiplied by the figure allotted to that stage and these results were summed to give a value for ZI.

RESULTS

Production of zygospores by Rhizopus sexualis *at* 20°

Progametangia (stages A and B) developed at the centre of colonies of *Rhizopus sexualis* on malt agar at 20° about 40 hr. after inoculation. Delimitation of the gametangia (stages C and D) took place 8–10 hr. later and a few mature zygospores (stage G) were present after a further 24 hr., i.e. approximately 72–74 hr. after inoculation (Table 1, column 6).

Table 1. *The effect of temperature on growth and zygospore formation in* Rhizopus sexualis

Treatments I–V, transferred from 20° to 7° at 24, 28, 32·5, 43·5 and 50·5 hr. after inoculation, respectively. The entries in heavy type indicate the time at which transfer was made. Treatment VI, kept at 20° throughout experiment.

Figures represent average diameter (cm.) of colonies. (f) = flat colony, hyphae creeping on surface of medium. (a) = upright aerial hyphae present.

A–G represent stages in development of zygospores (see Fig. 1), coefficients are numbers of each stage present in arbitrarily selected microscopic fields (see p. 759, 760).

P = total number of pairings in these fields.

ZI = quantitative index of zygospore production (see p. 761).

Period after inoculation (hr.)	Treatments					
	I	II	III	IV	V	VI
24	2·7 (f)	2·7 (f)	2·6 (f)	2·6 (f)	2·7 (f)	2·6 (f)
28	2·7 (f)	2·9 (f)	3·0 (f)	2·9 (f)	2·9 (f)	2·9 (f)
32·5	2·9 (f)	3·0 (f)	3·4 (a)	3·3 (a)	3·3 (a)	3·3 (a)
43·5	3·0 (f)	3·3 (f)	3·7 (a)	4·5 (a) (15A, 11B, 2C, 2D, 1E, 5F) P = 36 ZI = 86	4·6 (a) (8A, 14B, 3C, 2D, 1E, 4F) P = 32 ZI = 82	4·7 (a) (7A, 17B, 2D, 3E, 3F) P = 32 ZI = 82
50·5	3·1 (f)	3·4 (f)	3·9 (a)	4·7 (a) (10A, 10B, 3C, 3D, 4E, 7F) P = 37 ZI = 113	5·3 (a) (3A, 13B, 4C, 2D, 4E, 20F) P = 46 ZI = 189	5·4 (a) (1A, 4B, 13C, 3D, 17F) P = 38 ZI = 168
67·5	3·2 (f)	3·5 (f)	4·0 (a)	4·9 (a) (9A, 3B, 2C, 3D, 2E, 11F, 7G) P = 37 ZI = 158	5·5 (a) (3A, 8B, 1C, 1D, 7E, 18F, 7G) P = 45 ZI = 217	7·4 (a) (6A, 24B, 5C, 4D, 5E, 33F, 1G) P = 78 ZI = 315
119·5	3·5 (f)	3·8 (f)	4·15 (a)	5·0 (a) (9A, 3B, 8F, 17G) P = 37 ZI = 182	5·6 (a) (3A, 3B, 10F, 30G) P = 46 ZI = 279	9·0 (a) (4A, 7B, 4C, 6D, 6E, 16F, 64G) P = 107 ZI = 628

Transfer to low temperature at various intervals after inoculation

In a typical experiment, 18 malt agar plates were given a preliminary incubation at 20°. Batches of three plates were transferred to 7° after 24, 28, 32·5, 43·5 and 50·5 hr., respectively. Table 1 shows diameter of colonies, type of growth, total numbers of conjugations (P), numbers of conjugations at each stage (Fig. 2) and the zygospore index (ZI), at intervals during the experiment.

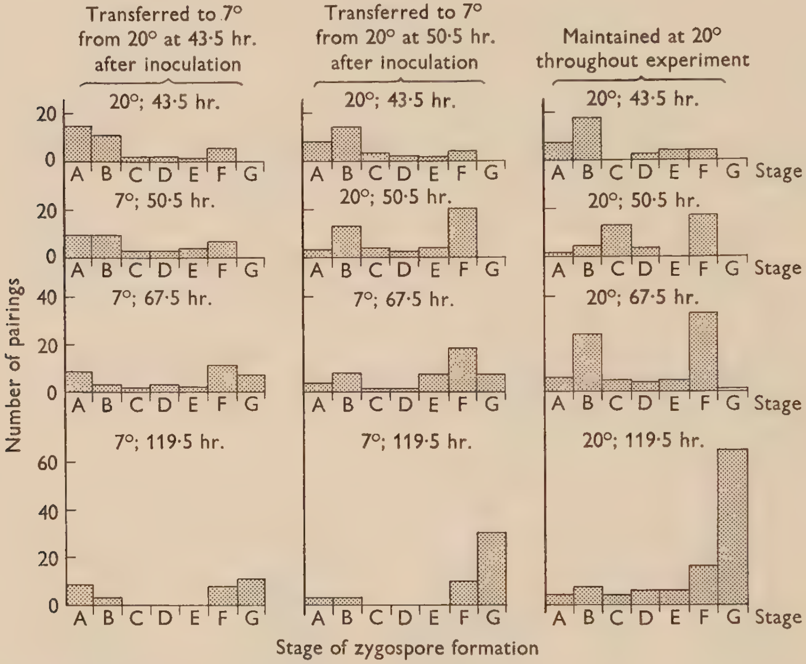

Fig. 2. Effect of transfer from 20° to 7° on development of zygospores of *Rhizopus sexualis*. Each block represents number of zygospores at the particular stage A–G (Fig. 1) at intervals after inoculation.

No gametangial initials developed after transfer to 7° in any culture on which they were not already present at the time of transfer, i.e. treatments I, II and III of Table 1. Even where (as in cultures subjected to treatment III) aerial hyphae, which would have borne gametangia if left at 20°, had already developed, no conjugations took place at the low temperature. In colonies transferred at a later stage, i.e. treatments IV and V, conjugation had already begun. Zygospores which had reached stage C, or a later stage, continued to develop at 7°, but only a few of those at earlier stages reached the next one. These few were presumably already physiologically near the next stage of development. In other experiments the effect of transfer of colonies from 20° to 5°, 10° and 11·5° was studied. Fig. 3 shows the fate of particular pairs of progametangia or gametangia as a result of these treatments.

The results of these experiments and of experiments with slide cultures may be summarized as follows:

(1) The formation of aerial hyphae which normally precedes zygospore formation is inhibited at low temperatures. Aerial hyphae which have already formed continue to develop.

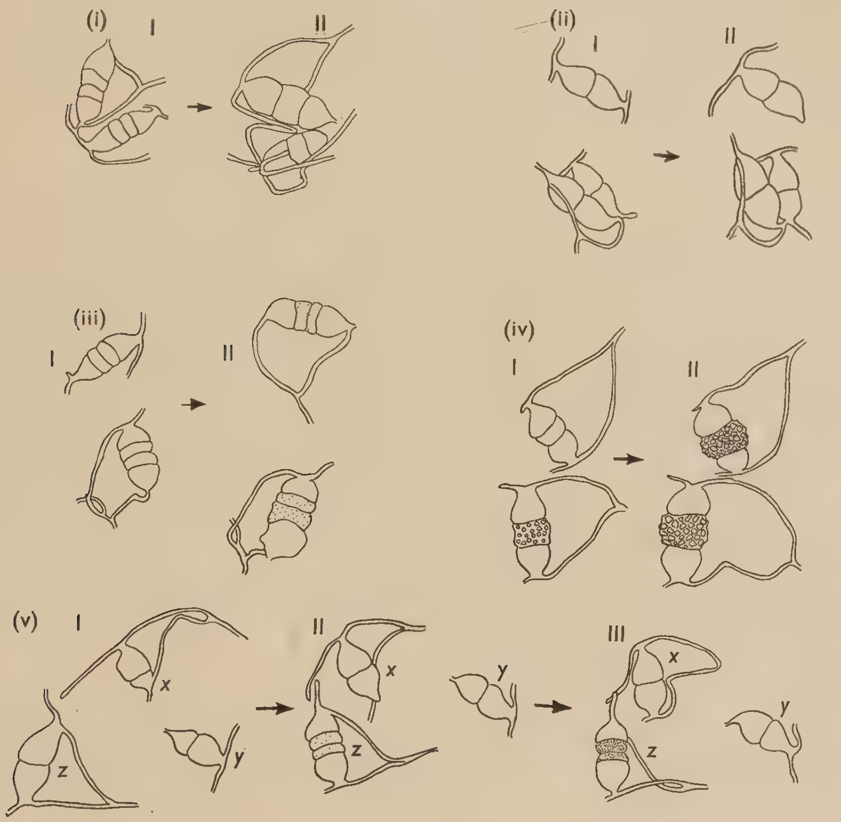

Fig. 3. Effect of exposure to 10° on formation of zygospores by *Rhizopus sexualis*. (i), (ii), (iii) and (iv), the condition of pairs of progametangia and gametangia: I, immediately before transfer to 10°; II, 48 hr. later. ×45. (v) Three pairs of progametangia, x, y and z: I, before transfer to 10°; II, after 24 hr. at 10°; III, after a further 48 hr. at 10°. ×45.

(2) Gametangia are not formed at 5–10° unless visible progametangial initials are present when the colonies are transferred to the low temperature, i.e. low temperature prevents the initiation of conjugation. Temperatures of 11·5° and over do not inhibit the formation of progametangia.

(3) Progametangia (stages A and B) present on colonies at the time of transfer do not develop further at 5°, but may occasionally do so at 7° or 10°. This tendency to continue development is increased in the presence of mature zygospores at the time of transfer (see columns IV and V of Table 1). Progametangia continue to develop normally at temperatures of 11·5° and higher.

(4) When stages C and D have been reached before transfer to low temperature, development usually continues at a decreased rate. Abnormal zygospores (stage X, Fig. 1H) increase in frequency with decrease in temperature.

(5) When colonies which have been exposed to low temperature are restored to 20°, development of initials already present and initiation of new progametangia are resumed, showing that the low temperature produces only a temporary inhibition of zygospore initiation and development.

(6) Sporangia are produced regularly but sparsely at 20°. After about 3 weeks at low temperature, sporangia are abundant and their formation is independent of the presence of zygospores.

The effect of brief exposures to 20°

It seemed possible that exposure to higher temperatures for the time needed to make observations in the experiments described in the previous section might have influenced results. This was tested by exposing cultures to a temperature of 20° for varying intervals during cold treatment. It was found that the low-temperature effect was not reversed by less than 4 hr. at 20°.

The effective time of exposure to 20° varies according to the age of the colony. When aerial hyphae are present whilst the colony is still at low temperature, exposure for 4 hr. to 20° is sufficient to initiate a few young stages. These do not mature when the cultures are replaced at low temperature but develop only as far as stage X. A longer time at 20° is required between the cold periods for fully mature zygospores to result. Exposure for 8 hr. at 20°, followed by resumed cold treatment, resulted in a number of zygospores which ultimately matured at low temperature. Exposure for 12 hr. to 20° gave similar results, with a great increase in the number of zygospores as compared with those formed on colonies given 8 hr. at 20°.

Continuous exposure to 20° after cold treatment frequently results in rapid development of, or increase in, aerial hyphae on that part of the colony which grew before or during cold treatment. This suggests that some product accumulates at low temperature and becomes available on return to 20°, resulting in this great aerial growth.

Clearly there is no permanent effect of cold treatment on the fungus. Hyphal tips from cultures grown at 7° were subcultured and incubated at 20°, when they showed a growth rate comparable to that of tips from cultures grown at 20°.

The influence of composition and pH *value of the medium on the response of* Rhizopus sexualis *to low temperatures*

In addition to the 1·0 % (w/v) malt extract agar used for most experiments, the following agar media were tested: 2·0, 3·0 and 5·0 % (w/v) malt, yeast extract, carrot extract, prune extract, glucose + asparagine, glucose + ammonium salts and glucose peptone. The time for progametangial initials to appear, and the time for mature zygospores to develop varied considerably with different media at 20°. Inhibition of early stages of zygospore formation occurred at low temperatures on all the media tested, as on malt agar, but with carrot extract agar there was a slight increase in the number of young stages which continued to develop at low temperature.

It seemed possible that low temperature might act by influencing changes in the pH value of the medium. *Rhizopus sexualis* forms zygospores over the range 3·0–8·0 of initial pH values. Varying the initial pH value of the medium over this range did not prevent the inhibition of initiation and early development of zygospores at low temperatures.

The effect of low temperature on other species

Phycomyces blakesleeanus. No inhibition of initiation or maturation of zygospores occured at 7°, or higher, but exposure to 5° decreased the number of new progametangia which arose (Table 2).

Table 2. *The effect of low temperature on zygospore production in five species of the Mucorales*

The colonies were incubated at 20° until they had reached the stages indicated in the column headings and were then transferred to the low temperature. The results given were obtained after 4 days at the low temperature.

The first symbol in each column refers to the degree of inhibition of new progametangia, the second refers to the degree of inhibition of further development and maturation of conjugations which had reached stages A–C at the time of transfer. 0 = complete inhibition; (0) = occasional initiation of new progametangia; (+) = development continues but is not completed; (X) = occasional production of abnormal zygospores; + = no inhibition.

	Condition of colony on transfer to low temperature			
Species	No mature zygospores		Mature zygospores present	
Temperature 5°				
Rhizopus sexualis	0	0 (X)	0	+
Mucor hiemalis	0	0	0	+
Zygorrhynchus macrocarpus	0	0	0	(+)
Z. moelleri	0	0	0	(+)
Phycomyces blakesleeanus	(0)	+	+	+
Temperature 7°				
Rhizopus sexualis	0	+	0	+
Mucor hiemalis	0	0 (X)	0	+
Zygorrhynchus macrocarpus	0	(+)	0	+
Z. moelleri	(0)	+	(0)	+
Phycomyces blakesleeanus	+	+	+	+
Temperature 10°				
Rhizopus sexualis	0	+	(0)	+
Mucor hiemalis	+	+	+	+
Zygorrhynchus macrocarpus	+	+	+	+
Z. moelleri	+	+	+	+
Phycomyces blakesleeanus	+	+	+	+

Mucor hiemalis. Zygospores were not produced at 5° or 7° when conjugation had not taken place before transfer to the low temperatures, but when older stages were present before transfer, zygospores developed from pre-existing initials (stage B for 7°, stage D for 5°). No new progametangia were initiated at 7° or 5°. There was no inhibition of initiation or development at 10° (Table 2).

Zygorrhynchus macrocarpus. At 5° initiation and maturation of progametangia and zygospores was inhibited unless a number of mature zygospores were present, in which case pre-existing progametangia (stage B) developed until partially mature but did not attain full maturity, reaching only stage F, with some pigmentation. At 7° initiation was similarly inhibited, but pre-existing conjugation matured as far as stage F only. When a number of mature zygospores were present, however, the development of younger ones continued to full maturity. There was no inhibition of initiation or development at 10° (Table 2).

Zygorrhynchus moelleri. Initiation of progametangia never occurred at 5° and rarely at 7°. At 5° pre-existing conjugations (stage B) continued to develop but did not mature completely, whilst at 7° maturation was eventually completed at the low temperature. No inhibition occurred at 10°.

DISCUSSION

It is clear that, while the whole process of conjugation and zygospore formation is slowed by a lowering of temperature below the optimum, the effect of low temperatures is greatest at certain early stages in this process. The actual temperature at which inhibition of conjugation and zygospore development occurs varies with the species but the differential effect of temperature on early stages was seen in all five species examined. This greater sensitivity to low temperature at early stages in zygospore development suggests either a temperature-induced block in the synthesis of some substance or substances essential to further development, or a slowing of translocation of such substances into the gametangia from the supporting hyphae.

Under some circumstances the presence of mature zygospores in a colony at the time of exposure to the critical low temperature enables the continued development of young spores which would otherwise have been checked. Work now in progress indicates that this effect of mature zygospores is due to a substance or substances which diffuses from them. It is suggested that this may be identical with, or able to replace, an essential substance the synthesis of which is inhibited by low temperature. The effect of low temperature is thus more likely to be due to the inhibition of synthesis rather than a retardation of diffusion or translocation. The nature of the inhibitory effect of low temperature is being further examined.

REFERENCES

Baker, R. E. D. (1931). Spore formation in *Sporodonia grandis* (Link.). *New Phytol.* **30**, 303.

Barnett, H. L. & Lilly, V. G. (1950). Nutritional and environmental factors influencing asexual sporulation of *Choanephora cucurbiratum* in culture. *Phytopathology*, **40**, 80.

Callen, E. O. (1940). Morphology, cytology and sexuality of the homothallic *Rhizopus sexualis* (Smith) Callen. *Ann. Bot., Lond.* N.S. **4**, 791.

Hawker, L. E. (1950). *Physiology of Fungi.* University of London Press.

Hawker, L. E. (1957). *The Physiology of Reproduction in Fungi.* Cambridge Monographs in Experimental Biology. Cambridge University Press.

Hesseltine, C. W. & Anderson, P. (1956). The genus *Thamnidium* and a study of the formation of its zygospores. *Amer. J. Bot.* **43**, 696.

Lilly, V. G. & Barnett, H. L. (1951). *Physiology of the fungi.* New York: McGraw Hill.

Robbins, W. J. & Schmitt, M. B. (1945). Factor Z_2 and gametic reproduction by *Phycomyces. Amer. J. Bot.* **32**, 320.

Smith, G. (1938). New species of mould fungi. *Mucor sexualis. Trans. Brit. mycol. Soc.* **22**, 252.

(*Received* 15 *July* 1957)

Books Received

'Autonomic Nervous System.' Edited by W. S. Feldberg. London: The Medical Department of The British Council. *British Medical Bulletin*, 1957, **13**, no. 3, 77 pp. Price 20*s*.

Biological Aspects of the Transmission of Disease. Report of Symposium of the Institute of Biology. Edited by C. Horton-Smith. London: Published for the Institute of Biology by Oliver and Boyd. 1957, 184 pp. Price 21*s*.

Histological Technique. By H. M. Carleton and R. A. B. Drury. London: Oxford University Press. 1957, 343 pp. Price 30*s*.

Introduction to Bacterial Physiology. By C. E. Clifton. London: McGraw-Hill Book Company, Inc. 1957, 414 pp. Price 64*s*.

'Liver: some physiological and chemical aspects, The.' Edited by N. F. Maclagan. London: The Medical Department of The British Council. *British Medical Bulletin*, 1957, **13**, no. 2. 77 pp. Price 20*s*.

Microbial World, The. By R. Y. Stanier, M. Doudoroff and E. A. Adelberg, Englewood Cliffs, New Jersey, U.S.A.: Prentice-Hall. London agents: Bailey Bros. and Swinfen Ltd., 1957, 682 pp. Price 72*s*.

Stvdia Revista de la Universidad del Atlantico, Barranquilla-Colombia, **11**, nos. 11–12.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its twenty-fourth General Meeting in the Royal Institution, London, on Monday, Tuesday and Wednesday, 15, 16 *and* 17 *April* 1957. *The following communications were made:*

COMMUNICATIONS

Cysteineless Mutants of *Salmonella typhimurium* arising as a Result of an Apparent Chromosomal Deletion.

By R. C. Clowes (*Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W.*2)

The ability of any pair among a series of phenotypically-similar, auxotrophic mutants to recombine to produce prototrophs, requires the concept of the gene as a physiological unit to be an extended segment of genetic material, the mutations then being envisaged as occurring at different points along this functionally undifferentiated region.

Among a series of independently isolated mutants, most strains differ by this criterion, but certain pairs do not recombine. This absence of recombination is suggested to be a result of mutations, either at sites too near to separate by crossover, or at identical sites, the mutants being duplicates. Occasionally, mutants are found which do not recombine with most other mutants of the same phenotype, suggesting that the mutation in this instance extends over the greater part of the gene locus as a result of some chromosomal aberration (multiple-site mutant).

Genetic analysis, by transduction to prototrophy with lysogenic phage, of certain cysteineless mutants of Salmonella, shows that all these mutants can be transduced by mutants of other phenotypes. However, although eight mutants recombine in any combination, the remaining six recombine neither among themselves, nor with seven of the previous mutants, and appear to be multiple-site mutants. In four mutants (*cys* C group), thiosulphate or sulphite can replace cyst(e)ine, but the remainder (*cys* D group), including the multiple-site mutants, grow only on thiosulphate or cyst(e)ine. *Cys* C mutants are fed by certain *cys* D mutants, the syntrophically active strains corresponding to those behaving genetically as point mutants, and the non-feeders to those identified as multiple-site mutants.

The results are consistent with two adjacent loci, *cys* C and *cys* D controlling sequential steps in cysteine biosynthesis, viz. sulphate $\underset{\text{C}}{-+\rightarrow}$ sulphite $\underset{\text{D}}{-+\rightarrow}$ thiosulphate $--\rightarrow$ cysteine. The multiple-site mutants appear to have an apparent chromosomal deletion extending over part of the *cys* C and most of the *cys* D locus and are thus deficient of both functions controlled by these loci.

Prejudice in Bacterial Classification. By P. H. A. Sneath (*National Institute for Medical Research, London, N.W.7*)

Much of the prejudice found in bacterial classification is due to attempts to give greater importance to some features than to others. It is very difficult to lay down criteria for importance and in doubtful instances the dilemma is usually resolved by giving equal importance to every feature. In the method described below, this latter principle is assumed from the start, and although this may be itself a prejudice, it does avoid the difficulty of deciding relative importance. The basic procedure is to tabulate the number of similarities and the number of differences between pairs of strains and to express the overall similarity by an index S, which is (number of similarities) divided by (number of similarities plus number of differences). The strains may now be sorted into groups, each group consisting of very similar bacteria, and the taxonomic relationships of the groups may be obtained from the mean S values. The definition of a feature is arbitrary but consistent. The method can be adapted to a mechanical sorting procedure (e.g. by an electronic computer) and seems to give worth-while results as illustrated by an example with a collection of chromogenic bacteria. Fuller details will be published shortly.

What is *Leuconostoc citrovorum*? By Ellen I. Garvie (*National Institute for Research in Dairying, University of Reading*)

Leuconostoc citrovorum (Hammer) Hucker & Pedersen is the name given to the catalase-negative Gram-positive coccus which produces gas from glucose, but which does not ferment sucrose. Two cultures originating from Hammer are housed in the A.T.C.C. One of these, 8082, ferments sucrose; the other, 8081, has been placed in the genus *Pediococcus* by Felton & Niven. This second culture is presumably respresentative of the cultures originally called *Streptococcus citrovorus* by Hammer. A.T.C.C. 8081 requires folinic acid, the so-called ‘citrovorum factor’, for growth. There is no evidence that Hammer did in fact study the non-sucrose-fermenting species of the genus *Leuconostoc*, and none of the cultures passed on by Hammer to Hucker and Pedersen were of this species.

Sønke Knudsen & Sørensen did handle non-sucrose-fermenting strains of *Leuconostoc*; they, however, used Orla Jensen’s classification, and called their cultures *Betacoccus cremoris* (X form). The assumption made in 1931 by Hucker & Pedersen that *Streptococcus citrovorus* and *Betacoccus cremoris* were the same cannot be supported by evidence in the literature. A recent study of A.T.C.C. 8081 and cultures conforming to the description of *B. cremoris* shows many similarities, and it is easy to understand why 25 years ago the cultures were placed together.

It is suggested that the catalase-negative Gram-positive coccus which produces gas from glucose, and which does not ferment sucrose, should be called *Leuconostoc cremoris*, or, if the European classification is used, *Betacoccus cremoris*, and the name *Leuconostoc citrovorum* discarded. This might avoid confusion with the ‘citrovorum factor’.

The Differentiation and Classification of Bacteria by Paper Partition Chromatography. By A. T. R. Mattick, N. J. Berridge, G. C. Cheeseman and L. A. Mabbitt (*National Institute for Research in Dairying, University of Reading*)

When bacterial cells are hydrolysed and the resulting amino acids are determined there results a common background made up of most of the known amino acids, against which a few differences may be observed between different species. When, however, the whole cells are subjected to extraction by a suitable solvent the common background is much less, important and obvious differences in quantities of individual free amino acids appear, and interesting peptides can be observed. Intact bacterial cells were therefore washed and then extracted with 10% (v/v) acetic acid and the extract subjected to paper chromatography in two dimensions. Amino compounds were revealed with ninhydrin. The following bacterial species were among those examined: *Lactobacillus casei*, *L. plantarum*, *L. brevis*, *L. fermenti*, *Streptococcus lactis*, *S. cremoris*, *S. durans*, *S. faecalis*, and micrococci of different groups, some received from Dr S. T. Cowan, others from Dr T. Gibson. Every species gave a chromatographic pattern of spots and streaks different from that of every other species, but different strains within a species gave patterns with common properties.

To establish the degree of constancy of pattern within species we examined 28 strains of *L. casei* and 30 of *L. plantarum*, previously classified by Dr M. E Sharpe, using serological and biochemical methods. Six distinguishing properties readily seen on chromatograms (e.g. presence or absence of proline) were discovered. Fifty-three of the 58 strains had 5 or 6 properties 'correct', 4 had two 'incorrect', and only one strain appeared intermediate, with three properties of each kind.

Ribonucleoprotein-containing Structures in the Cytoplasm of a Parasitic Flagellate. By B. A. Newton (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Basophilic, rod-like particles have been observed in the cytoplasm of the flagellate *Herpetomonas muscae* by Wenyen (1913) and Becker (1923), who suggested that they were nuclear fragments which only occurred in degenerating organisms; the present communication describes similar structures occurring in the cytoplasm of a closely related organism, *Strigomonas* (*Herpetomonas*) *oncopelti*. Rod-like particles have been observed in unfixed preparations of *S. oncopelti* by phase-contrast microscopy; organisms contain either one or two of these structures which are always located at the posterior end of the cell. They are present in organisms harvested at all stages of growth in either peptone-glucose medium or a simple synthetic medium (Newton, 1956). Starvation of organisms by incubation in a buffered salts medium in the presence or absence of phosphate does not result in the loss of these structures.

The results of cytochemical tests suggest that ribonucleic acid (RNA) is associated with the particles: they are stained blue by May-Grunwald & Giemsa dyes and red by pyronin G, and the material stained by either of these methods is removed by treatment with ribonuclease. There is no evidence for the presence of polysaccharide, lipid, metaphosphate or deoxyribonucleic acid.

Particles have been liberated from cells by shaking suspensions of organisms in 2·5 M-sucrose with small glass beads and then separated from other cell components by centrifugation through a medium of graded sucrose concentration. Analysis of isolated particles shows that they are composed largely of ribonucleoprotein and contain *c.* 10–20 % of the total RNA of whole organisms. Electron microscope studies of isolated particles and of ultra-thin sections of whole organisms show that these rod-like structures are bounded by a thin membrane and that they probably divide by transverse fission.

REFERENCES

BECKER, E. R. (1923). *J. Parasit.* **9**, 199.
NEWTON, B. A. (1956). *Nature, Lond.* **177**, 279.
WENYEN, C. M. (1913). *Arch. Protistenk.* **31**, 1.

Properties of an *Eremothecium ashbyi* Strain-producing Riboflavin.

BY J. STÁRKA (*Department of General Microbiology, Charles University, Prague*)

Four phases can be observed during the cultivation of *Eremothecium ashbyi* in shaken culture on glucose-peptone-yeast autolysate medium. In the first phase (0–36 hr.) the maximum cell dry wt. is reached when all glucose is utilized. A significant quantity of fat (*c.* 35 % cell dry wt.) is formed, the riboflavin contents, however, remain very low (*c.* 10 μg/mg. cell dry wt.). The respiration of washed cells on glucose reaches its maximum after 24 hr., but the activity of catalase is slight. The beginning of the second phase (36–60 hr.) is characterized by sporulation followed by rapid decrease of respiration activity on glucose and a considerable increase of catalase activity. Simultaneously large amounts of riboflavin are synthetized mainly in bound form in the cell as FMN and FAD in contrast to the medium where only free riboflavin appears. The contents of fat drop slowly. In the third phase (60 hr.–7 days) the activity of catalase reaches its maximum on the third or fourth day and a large quantity of glycogen and volutin is being stored in the cells. The maximum of volutin coincides with the maximum of metaphosphate contents and with that of riboflavin. Both '7 min. phosphorus' and 'nucleic acid phosphorus' increase up to the sixth day. The maximum rate of riboflavin synthesis could be observed on the fourth or fifth day. Significant amounts of flavines are released from the cells into the medium where they are partly present in nucleotide form. In the fourth phase all variables that were followed up reach equilibrium.

The results obtained show that the synthesis of riboflavin occurs in the period when the growth of cells ceases and that the formation of flavines is correlated with a metabolic shift from cytochrome to flavinoxidase respiratory pathway.

Metallo-enzymes in Nitrite Reduction in *Neurospora*. By Antonia Medina and D. J. D. Nicholas (*A.R.C. Unit of Plant Nutrition (Micronutrients), Long Ashton Research Station, University of Bristol*)

The reduction of nitrate to ammonia is mediated by three enzymes, nitrite, hyponitrite and hydroxylamine reductases, present in cell-free extracts of *Neurospora crassa* macroconidial Wild type 5297 a. The enzymic activities of the enzymes were determined by the formation of ammonia, in Conway units, from nitrite, hyponitrite and hydroxylamine respectively, in the presence of DPNH. All reagents used were either boiled to eliminate the last traces of ammonia or dispensed in ammonia-free water. Sodium hyponitrite and hydroxylamine hydrochloride were freshly prepared daily. Nitrite reductase may also be determined by measuring nitrite disappearance after adding DPNH.

The three enzymes are flavin-dependent since FAD or FMN stimulate them and their inhibition by atabrine is reversed with FAD. Metal inhibitors reduce the activity of the three enzymes. The depressing effects of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and naphthaquinone on nitrate and hyponitrite reductases suggests the participation of a haem type compound, possibly cytochrome *b*, in the two enzymes. In considering ammonia production by the three enzymes in metal-deficient felts, inhibition of hydroxylamine or hyponitrite reductase would result in a depression in nitrite reductase provided the reductive pathway involved these intermediates. Mn deficiency only depressed hydroxylamine reductase and deficiencies of either Cu or Fe reduced nitrite and hyponitrite reductases. Inhibition of the three enzymes by *p*-chloromercuribenzoate was completely reversed by glutathione. Dinitrophenol depressed nitrite and hyponitrite reductases only. The aliphatic sequence of nitrate reduction in *Neurospora* is probably:

Oxidation state for N	+5		+3		+1		−1		−3
Reduction sequence	nitrate	→	nitrite	→	hyponitrite	→	hydroxylamine	→	ammonia
Metal		Mo		Fe Cu		Fe Cu		Mn	
Phosphorylation		—		P		P		—	

New Aspects of the Structure of the Yeast Cell Wall. By A. A. Eddy and A. D. Rudin (*Brewing Industry Research Foundation, Redhill*)

Previous workers have shown that the yeast cell wall consists largely of mannan and glucan together with smaller amounts of protein and lipid. The present study is concerned with (*a*) the nature of the ions at the yeast surface, and (*b*) the effects of various enzymes on the cell wall. It represents an approach to the problem of determining how the above components are linked and, further, how the agglutination characteristics of 'top' and 'bottom' yeasts depend on the respective compositions of the cell walls.

The electrophoretic mobility of washed cells of different strains of brewer's and baker's yeast was found to vary from $+0\cdot5$ to $-1\cdot5\,\mu\text{s.}^{-1}\text{V.}^{-1}\text{cm.}$ in measurements at constant ionic strength ($I=0\cdot005$) and various acidities in the range from pH 2 to 9. The charge density is therefore of the relatively low order of one electron per 10,000 Å^2.

The mobility of intact cells and of cell walls isolated from them varied with pH in a manner characteristic of the strain of yeast. In each case at least three types of ion were apparently involved but in different proportions, the contribution from the first type being independent of pH over a wide range. The ion itself is identified as bound phosphate on the evidence of a direct relationship between the respective phosphorus contents of the cell walls of different strains of yeast and their mobility at pH 4·0. The two other types of ion involved, tentatively attributed to the presence of protein at the wall surface, gave rise to sharp changes in mobility between pH 3 and pH 6 with charge reversal at about pH 4.

Enzyme preparations of papain, malt and juice from the snail gut were each found to dissolve the isolated yeast cell wall and, less readily, the corresponding wall of whole yeast. Using cell walls the reaction was initially fast but soon slowed and only in the case of the snail juice did the whole wall dissolve. Both the latter enzyme and the malt enzyme, but not papain, liberated free glucose from the wall. In all three cases mannan and glucan fractions were subsequently isolated from solution. At a stage in the digestion by papain, when the surface charge due to bound phosphate was 20 % of the initial value, the mannan fraction was shown to contain the greater part of the phosphate released by the enzyme. The evidence suggests that at least part of the mannan is located near the outer surface of the cell wall where it may form a mosaic with glucan.

The Composition and Distribution of Rough Somatic Antigens in some Gram-negative Bacteria.

By D. A. L. Davies (*Microbiological Research Establishment, Porton, Wiltshire*)

Pasteurella pestis does not possess an 'O' somatic antigen and is therefore Rough in the Salmonella sense. The main antibody response to this organism is directed against its protein antigens, one of which is responsible for smoothness of colonial morphology; this protein is not the envelope material. A polysaccharide produced by *P. pestis* (glucosamine, glucose, aldoheptose) belongs to the class of Rough somatic antigens.

In *Pasteurella pseudotuberculosis* there are 5 serological groups; group specific Smooth 'O' somatic polysaccharides have been obtained from representatives of each of these groups. These materials all contain deoxy-sugars, including tyvelose or abequose. Rough (avirulent) strains possess a polysaccharide closely related in composition and specificity to that of *P. pestis*. These Rough somatic antigens cross-react serologically under certain conditions with the Smooth somatic antigens, but no evidence could be found for the presence of a Rough polysaccharide as a separate chemical entity underlying the Smooth component in Smooth organisms, although this is the classical view.

The Smooth 'O' somatic antigen of *Shigella dysenteriae* is a well-characterized material (containing glucosamine, galactose, rhamnose) for which diethyleneglycol is a relatively specific solvent. Extraction of Rough (avirulent) *S. dysenteriae* with diethyleneglycol yields no polysaccharide but using the phenol extraction method of Westphal, a polysaccharide can be obtained (glucosamine, galactose, aldoheptose) which does not cross react with the products of Smooth cultures. When diethyleneglycol extracted Smooth cells are recovered and subsequently extracted by the phenol method no Rough polysaccharide can be isolated, a situation similar to that found in *P. pseudotuberculosis*.

It is suggested that the S → R change may reflect a change of polysaccharide composition, including a loss of deoxy sugars, rather than the loss of a discrete antigen.

Antigens of *Pasteurella septica*. By A. P. Maclennan and C. J. M. Rondle

(*Microbiological Research Establishment, Porton, Wiltshire*)

Analysis of the different species of *Pasteurella* by the agar diffusion precipitin method of Ouchterlony using sera prepared against organisms of each species, reveals that the several immunological types of *Pasteurella septica* which have been distinguished by means of passive protection tests have at least 6 antigens in common which are, however, absent from other *Pasteurella* species.

Absorption of an antiserum prepared against a type V strain of *Pasteurella septica* with the organisms of a different type discloses a number of type specific antigens. One of these antigens is serologically identical with a pyrogenic lipopolysaccharide, isolated by phenol extraction of acetone dried cells and found to contain glucosamine and an aldoheptose. The chemical composition of this lipopolysaccharide resembles that of the Rough somatic antigen of *P. pestis*, although the substances are serologically distinct.

Strains of one immunological type and apparently identical in antigenic composition may vary widely in virulence. Furthermore, loss of capsule accompanied by almost complete loss of virulence can occur without detectable antigenic change. In some dissociative processes the loss of capsule and virulence is accompanied by the appearance of autoagglutinability of saline suspensions. Where this occurs the lipopolysaccharide antigen may still be present in the autoagglutinable variant. On the other hand, two strains of different immunological type and possessing lipopolysaccharides of different serological specificity, gave rise to autoagglutinable variants in which these antigens could not be detected but which manifested a new specificity common to the two variants and based on a thermostable component of the cell. This antigen, detected by preparation of antisera to each of the variants, was absent from the virulent parent strains.

Endogenous Metabolism in *Sarcina lutea*. By E. A. Dawes and W. H. Holmes (*Biochemistry Department, Glasgow University*)

When a suspension of *Sarcina lutea*, harvested from peptone medium, is briskly aerated the Q_{O_2} (endogenous) falls rapidly while the Q_{O_2} (glucose) increases. Analysis of 'endogenous-lowered' cells shows that polysaccharide is not the endogenous substrate, and changes in Q_{O_2} indicate that, while endogenous respiration (ER) is unaffected by exogenous glucose, high levels of ER suppress glucose oxidation. This suppression was confirmed by oxidation of glucose-U-^{14}C when the evolution of $^{14}CO_2$ increased as ER diminished. That ER is independent of glucose metabolism was proved by a manometric technique. Some 70% of the radioactivity oxidatively assimilated from glucose-U-^{14}C was found in the carbohydrate fraction of the cells. The assimilated reserve is thus differentiated from the endogenous reserve.

Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) was investigated as an index of endogenous metabolism and found to diminish with ER during aeration. Incubation of 'endogenous-lowered' cells with glucose rapidly increases the TTC-value which attains a maximum while the ER is still increasing. Furthermore, the TTC-value is restored to its original level but the Q_{O_2} (endogenous) increases only slightly to reach a maximum far below that of the original cells. If incubation with glucose is continued after the TTC-value has been restored, cellular polysaccharide increases.

It is concluded that *Sarcina lutea*, during growth, lays down a non-polysaccharide reserve which, when later metabolized, can suppress the oxidation of exogenous substrates. This material cannot be replaced by the oxidative assimilation of glucose which causes the accumulation of polysaccharide. A pool of intermediates which can reduce TTC is associated with the metabolism of both types of reserve material.

Stimulation by X-radiation of Enzyme Induction and Growth in *Escherichia coli*. By H. Laser and Margaret J. Thornley (*Molteno Institute, University of Cambridge, and Low Temperature Research Station, University of Cambridge and Department of Scientific and Industrial Research*)

Escherichia coli B has been grown in a liquid medium containing, besides inorganic salts, glucose and ammonium chloride as sole sources of carbon and nitrogen. The cells do not grow if maltose is substituted for glucose. Similarly, washed cell suspensions which oxidize glucose vigorously have an insignificant O_2-uptake with maltose. However, maltose is utilized for growth if NH_4Cl is replaced by glutamic acid. Apparently the cells cannot form the enzyme needed to utilize maltose from their existing protein equipment but can if given a suitable organic nitrogen source.

X-radiation of 4–8 kr. (190 kVP., 10 mA., dose rate 8 kr./min.) changes these conditions fundamentally:

(i) Washed suspension of irradiated cells oxidize glucose as fast as controls but—unlike the controls—develop an increasing respiration in presence of maltose, the maximal rate (after 4–8 hr.) being attained within 3 hr. and approaching that for glucose.

(ii) This response to maltose is suppressed by chloromycetin.

(iii) Cells viable after irradiation can grow on maltose in the liquid medium with NH_4Cl, although slowly.

(iv) The viable cells with maltose and glutamate multiply much faster than unirradiated controls for about 8 hr. where the stationary phase is attained in about 32 hr. This has been confirmed by turbidity measurements and plate counts.

(v) The response to maltose is lost on incubation of the cells with glucose and NH_4Cl.

(vi) Both controls and irradiated cells contain maltase. This has been demonstrated with ultrasonically disintegrated cells where the addition of maltose and glucose oxidase (notatin) caused an O_2-uptake at equal rates in both samples.

These results and further evidence justify the conclusion that the observed irradiation effects are due to the induction of the transporting enzyme for maltose in irradiated cells.

Polarographic Estimation of Oxygen in Semi-solid Agar. By E. J. K. Penikett (*Guy's Hospital Medical School, London, S.E.* 1)

Mycobacterium tuberculosis was cultured in test tubes containing semi-solid agar medium to a depth of 4 cm. When incubated in air visible growth occurred in a band near the surface. If the partial pressure of oxygen was increased to 95 % the band of growth commenced about 2 cm. below the surface. It was thought that a gradient of oxygen concentration in the tubes would account for this phenomenon.

It has been found possible to measure molecular oxygen concentrations at different levels with a dropping mercury electrode.

In a 10-day Kirchner semi-solid agar culture inoculated with 100,000 organisms per millimetre, the molecular concentration of oxygen measured polarographically was found to be 3·0 ml./l. at the level at which growth commenced. The value was the same whether the culture was incubated in air or in 95 % oxygen. The concentration of oxygen at the surface in the tubes incubated in air was 3·1, and 8·6 ml./l. at the surface in the tubes incubated in 95 % oxygen.

The Effect of Oxygen on Growth of *Mycobacterium tuberculosis* in Semi-solid Agar. By R. Knox, A. J. Lister and C. G. A. Thomas. (*Guy's Hospital Medical School, London Bridge, S.E.* 1)

The tubercle bacillus is distinct from nearly all other pathogens of medical importance in being a strict aerobe. In previous work in this laboratory it has been shown (Knox, 1955; Knox, Swait & Woodroffe, 1956) that rapid growth of *Mycobacterium tuberculosis* occurs in deep culture in tubes of liquid medium such as Kirchner or Fisher made semi-solid by adding a small amount of agar (0·125 %). Preliminary experiments have shown an interesting relation between the gas phase, inoculum size and the amount of growth. It was therefore decided to investigate the effect of various mixtures of oxygen, nitrogen and CO_2 on the growth of *M. tuberculosis* in media of this type.

It was found that whereas in air growth occurred at and a few centimetres below the surface, in increasing concentrations of oxygen growth occurred only below the surface. In 95 % oxygen growth was completely suppressed when small volumes of medium were used but occurred below the surface in longer columns of medium. The organisms could recover from the suppressive effect of oxygen if they were removed from it after less than 7 days. The inhibitory effect of oxygen was most obvious with small inocula. With large inocula oxygen, instead of suppressing, strongly stimulated growth. Clearly there was an equilibrium between supply and demand.

The inhibitory effect appears to be due to molecular oxygen itself and various explanations are being investigated. One interesting lead is given by the fact that fresh, but not heated, catalase can reverse the effect of oxygen. It is known that isoniazid resistant strains of *Mycobacterium tuberculosis* are deficient in catalase and are much more sensitive to H_2O_2 than catalase positive isoniazid sensitive strains. Attempts are being made to find out whether isoniazid sensitive and resistant strains show any consistent differences in sensitivity to oxygen and whether this itself is in any way related to sensitivity to H_2O_2.

Actinophages and Lysogenic Actinomycetes in Nature. By M. Welsch (*Université de Liège*)

Actinophages have been known for the last 20 years, but their importance was not realized until 1947, when economically disastrous air-borne infections were observed in streptomycin production-plants.

We undertook to make a survey of the occurrence of actinophages in natural substrates. Liquid natural substrates, or aqueous extracts of solid ones, were sterilized by filtration, mixed with warm melted agar, and poured in Petri dishes. The hardened medium was next streaked with several indicator strains of streptomycetes. In some instances, discrete plaques were seen after incubation. Their number gave a rough estimate of the amount of phage present and viruses were easily isolated from them by standard procedures.

When negative results were recorded, enrichment was attempted. The natural substrate was suspended in water and seeded with a heavy suspension of conidia from one or several actinomycetes. After incubation on a shaker during 2–4 days, the supernatant liquid was decanted and tested as above.

These procedures have shown that several strains of actinophages, differing by the morphology of their plaques, their host-range, their antigenic properties, can be found in a single natural substrate. A given actinophage may be present at the titre of 10^3 to 10^4 particles/ml. in its natural habitat.

A search was also made for truly lysogenic streptomycetes. By testing the thymol-sterilized culture fluids of many strains upon many indicator strains, we observed that about 15 % of the organisms freshly isolated or taken from a collection of cultures actually carried a phage.

That it was true lysogeny was demonstrated by the constancy of the ratio phage/bacteria in different cultures, and by the maintenance of phage production after repeated single colony isolation or serial cultivation in a medium containing a specific anti-phage serum.

All the viruses so far isolated, whether virulent or temperate, act specifically upon streptomycetes of the *Streptococcus griseus* group.

Mycostasis in Soils. By C. G. Dobbs, W. H. Hinson, and Joan Bywater (*University College of North Wales, Bangor*)

W. H. Hinson (1950–4) showed that mycelia growing on protected surfaces of glass slides buried 14 days in soil developed mainly from spores germinating in condensed water on the glass, whereas spores in the soil did not germinate without nutrient. He found no exceptions to total inhibition of germination of spores of *Penicillium frequentans* on thin cellulose film in close contact with various moist surface soils. This inhibition decreased with depth and in some subsoils was absent at 6 ft. It was absent also from deep mineral material, and appears broadly to co-exist with biological activity in the soil. So far no fungus of which the spores germinate readily in distilled water has been found to be unaffected.

The inhibition is removed by addition of glucose to the soil above a threshold quantity which varies widely; also by heat, desiccation, or any other treatment liable to release nutrient by killing soil organisms; also by organic solvents and by mixing the soil with charcoal. It returns when the glucose has been attenuated by the soil organisms, and when the solvent has evaporated. Seitz-filtered water extracts of soils reduce germination but the effect diminishes with storage of the extract.

Recent work by J. Bywater, using *Mucor ramannianus*, has shown that percentage germination on an open cellulose film can be used for comparing the intensity of the effect in some soils. Others inhibit completely unless glucose is added, whereas two forest humus samples stimulated growth and sporulation of moulds when incubated.

The phenomenon has some bearing on the interpretation of current studies in soil fungal ecology, both by plating out soils, and by the use of buried glass devices for the study of soil mycelia.

A Possible Example of Geographical Variation in Bacteriophage Sensitivity. By R. Spencer (*Humber Laboratory, Department of Scientific and Industrial Research, Hull*)

Seventy-nine strains of luminous bacteria identified as *Photobacterium phosphoreum* were isolated from the intestines of eighteen different species of fish caught in the North Sea and were shown by a range of morphological, cultural and biochemical criteria to form a very homogeneous group. Seven strains of luminous bacteria from Japanese marine sources were also found to form a homogeneous group, which was almost identical to the North Sea group. It was considered that the two groups belonged to the same species.

The two groups were tested for sensitivity to a bacteriophage isolated from sea water taken from the North Sea, against a member of the North Sea group of luminous bacteria (Spencer, 1955), and statistically significant differences in sensitivity were found. The great majority of the strains constituting the North Sea group were sensitive, whilst not one strain of the Japanese group was sensitive. The tentative conclusion is that this is most probably an example of geographical variation in bacteriophage sensitivity.

REFERENCE

Spencer, R. (1955). *Nature, Lond.* **175**, 690.

Potentiation of Serum Bactericidal Titre by Clinical Dextran.

By A. C. Wardlaw (*Wright-Fleming Institute of Microbiology, London*)

Dilutions of normal human serum made in 6 % (w/v) clinical dextran have a higher bacterial activity towards strains of *Escherichia coli* and *Shigella shigae* than do the same dilutions made in normal saline. Thus the 50 %-killing dilution of serum is increased by a factor of 5–8. If the dextran concentration is reduced to 1 % (w/v), the potentiating effect is lost. In contrast to clinical dextran, native dextran strongly inhibits serum bactericidal activity, while 6 % (w/v) glucose in saline gives the same result as saline alone. Bovine serum albumin, which has a molecular weight similar to that of clinical dextran, potentiates serum bactericidal activity only slightly.

Clinical dextran does not enhance the activity of haemolytic complement; furthermore, its presence in serum accelerates the inactivation of complement at 37°. It is therefore considered more probable that the effect of clinical dextran on serum bactericidal activity is due to a direct action on the organisms rather than on the serum factors. The nature of this action has not been determined. It has, however, been shown that the strains used undergo changes in their sensitivity to the lethal action of serum during growth in culture. Sensitivity is maximal during the log phase and minimal in the early lag phase.

The Occurrence of the Fertility Factor F in Wild Strains of *Escherichia coli*.

By G. Furness (*The Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, Paddington, London, W.2*)

A percentage of wild strains of *Escherichia coli* which did not recombine with *E. coli* K12$S^{R}F-$ mutants infected them with F.

Wild strains varied in their ability to transfer F to different K12$S^{R}F-$ auxotrophs. Recombinations in which the only difference was the origin of the F factor differed greatly in the percentage transfer of unselected markers. The order of the unselected markers, however, was unchanged and was in agreement with that given by other workers. This suggests that there are different F agents which affect the transfer of genetic material in recombination.

Followed by a Symposium on

MICROBIAL ECOLOGY

The Genome as a Component of the Ecosystem. By C. J. SHEPHERD (*University of Cambridge*)

Nutritional Aspects of Microbial Ecology. By JANE GIBSON (*University of Sheffield*)

Effects of Hydrogen-ion Concentration and Oxidation Reduction Conditions on Bacterial Behaviour. By L. F. HEWITT (*Serum Research Institute, Carshalton*)

The Role of Light in the Microbial World. By R. Y. STANIER and GERMAINE COHEN-BAZIRE (*University of California*)

Micro-organisms Resisting High Concentrations of Sugars or Salts. By M. INGRAM (*Low Temperature Research Station, Cambridge*)

The Action of Pressure and Temperature. By F. H. JOHNSON (*Princeton University*)

The Gaseous Environment. By W. VERHOEVEN (*Technische Hogeschool, Delft.*)

The Ecological Significance of Antibiotic Production. By P. W. BRIAN (*Akers Research Laboratories, Welwyn*)

The Relations of Bacteriophage to Bacterial Ecology. By E. S. ANDERSON (*Central Enteric Reference Laboratory, Colindale*)

The Predacious Fungi and their Place in Microbial Ecology. By C. L. DUDDINGTON (*The Polytechnic, London*)

Ecological Factors in the Survival of Fungi. By LILLIAN HAWKER (*University of Bristol*)

Some Factors in the Life of Free-living Protozoa. BY J. A. KITCHING (*University of Bristol*)

Ecology of Micro-organisms in Soil as Observed during their Development upon Buried Cellulose Film. By H. T. TRIBE (*University of Cambridge*)

The Role of Plant Hosts in Microbial Ecology. By F. C. BAWDEN (*Rothamsted Experimental Station*)

The Biological Influence of Man and Animals in Microbial Ecology. By R. LOVELL (*Royal Veterinary College, London*)

Ecology of Viruses. By C. H. ANDREWES (*National Institute for Medical Research, London*)

The Selective Action on Bacteria of Various Factors inside and outside the Animal Body with Particular Reference to their Effect on Virulence. By G. S. WILSON (*Public Health Laboratory Service, London*)

Some Factors Influencing the Spread of Plant Viruses by Arthropod Vectors. By K. M. SMITH (*Virus Research Unit, Cambridge*)

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